This volume will be completed with the forthcoming issue, Vol. 39, No. 5, *Proceedings for the 24th General Meeting of Japanese Biochemical Society* (May 1952)

Vol. 39, No. 4 **September, 1952**

THE JOURNAL
OF
BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

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THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

PRINTED IN JAPAN

The Journal of Biochemistry, Vol. 39, No. 4.

THE METABOLISM OF TRICHOMONAS VAGINALIS, WITH COMPARATIVE ASPECTS OF TRICHOMONADS

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(Received for publication, February 28, 1952)

In the previous papers (*1*, *2*) there were reported certain aspects of carbohydrate metabolism of *Trichomonas foetus* under both aerobic and anaerobic conditions. These investigations have added some new findings to our meagre knowledge of the biochemistry of trichomondads. However, little is known concerning the metabolism of *Trichomonas vaginalis*, so the authors carried out this metabolic investigation on this parasitic flagellate.

In order to elucidate the mechanism of chemotherapeutic action of drugs upon parasitic microorganisms, it is essential to make clear the metabolic features of these organisms. Especially, the authors should like to emphasize the importance of comparative biochemistry, for the chemotherapeutic potentiality depends mainly on its highly selective toxicity towards pathogens. The present study was undertaken with such an intension and the following results have been obtained.

MATERIALS AND METHODS

Trichomonads Suspension—The bacteria-free *T. foetus* (strain *Tricho*) parasites were obtained by the same method as previously described (*1*). The Izumida strain of *T. vaginalis* used was isolated in 1951 by Y. Hamada of our laboratory from the vaginal discharges of a patient at the Kitano Hospital, Osaka. The bacteria-free culture of *T. vaginalis* was carried out at 37° for 48 hours in boiled bovine liver extracts containing 1 per cent peptone, 20 per cont bovine serum, 1 per cent maltose, 0.1 per cent cysteine and 0.5 per cent NaCl (pH 5.6). The parasites were concentrated by gentle centrifugation. After being washed twice with 0.25 M NaCl solution, they were so resuspended in the saline solution that the latter contained about 10^8 cells per ml.

Measurements—Manometric measurements of oxygen uptake were performed at 37° by use of Warburg Manometer as described by Umbreit *et al.* (*3*). Catalase activity was measured by the method of Fujita *et al.* and represented as Q_{cat} (*4*).

RESULT

The Determination of Standard Conditions for the Study of Glucose Oxidation—The factors affecting the rate of oxygen uptake with glucose by *T. vaginalis* were investigated. The effect of pH upon the respiration is indicated in Fig. 1. In the initial pH range of 5.4–6.0 the oxygen uptake was maximal. Then, the effect of NaCl concentration in 0.05 *M* phosphate buffer at pH 6.0 was studied (Fig. 2). The optimal range of the concentration was 0.1–0.2 *M* NaCl. When pure oxygen or a gas mixture of 5 % O_2-95 % N_2 was substituted for air in the gas phase of Warburg flasks, the velocity of oxygen uptake varied according to oxygen tensions (Fig. 3). It is apparent that oxygen has an inhibitory effect on the respiration. On the basis of these preliminary tests, the standard conditions mentioned above were adopted for the manometric determination of oxygen uptake.

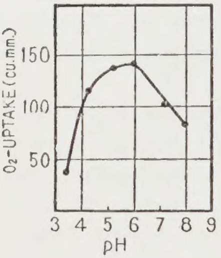


FIG. 1. Effect of pH upon oxygen uptake.

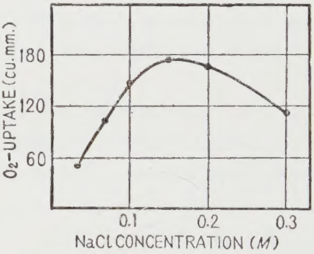


FIG. 2. Effect of NaCl concentration upon oxygen uptake.

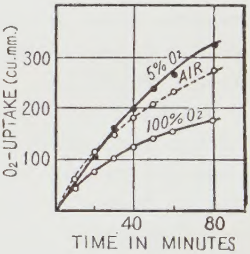


FIG. 3. Effect of oxygen tension upon oxygen uptake.

(Oxygen uptake were measured for 60 minutes in Figs. 1 and 2. 0.02 *M* glucose was used as substrate in all experiments.)

Substrates of the Respiration—To test the ability of *T. vaginalis* to oxidize various substrates, thirteen compounds were used as substrates. These substrates may be divided into three groups according to their utilizabilities by *T. vaginalis;* (a) Maltose and glucose were rapidly oxidized; (b) Lactate and pyruvate were oxidized at half so much a rate as glucose; (c) The following substances were not utilized at all:

succinate, citrate, DL-α-alanine, formate, acetate, butyrate, glutamate and gluconate. The results obtained with some of these substrates are shown in Fig. 4.

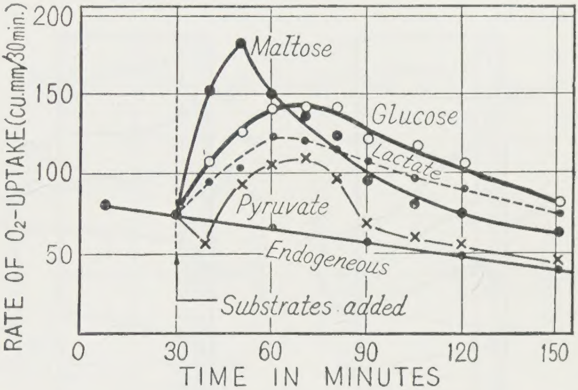


FIG. 4. The respiration of *T. vaginalis* on various substrates. The substrate was added from the side arm after 30 minutes to give final concentration of 0.02 *M*. Total volume, 2.2 ml. Temperature 37°. Gas phase, air. With 2.0 ml. KOH (20%) in center well.

The Effect of Inhibitors upon the Oxygen Uptake—The effect of several inhibitors upon the respiration was investigated. As shown in Table I, the respiration was highly sensitive to -SH reagents, such as iodoacetate, arsenite and *p*-chloromercuribenzoate (PCMB). On the other hand, cyanide, azide and malonate did not inhibit the respiration even in their high concentrations. Sodium fluoride exerted a relatively strong inhibition.

The Effect of Oxygen Tension upon the Respiration—In the preliminary tests, it was found that oxygen had a toxic effect upon the respiration of *T. vaginalis*. As for *T. foetus*, the rate of oxygen uptake was increased by 10 per cent when 100 per cent oxygen was used (*1*). Therefore, further comparative studies were carried out on this point.

Under various oxygen tensions, *i.e.*, 5% O_2-95 % N_2; 10 % O_2 90 % N_2; air; 50 % O_2-50 % N_2; and 100 % O_2, the oxygen uptake of *T. foetus* or *T. vaginalis* was measured with or without glucose, respectively. In the case of *T. foetus*. the respiration was somewhat stimulated by increasing oxygen tensions within 60 minutes. There occurred, however, a slight inhibition after 120 minutes under high tension (Fig. 5).

In the case of *T. vaginalis* the inhibitory effect of oxygen upon the glucose respiration increased with oxygen tension. After 60 minutes the oxygen uptake in 5 per cent oxygen became predominant over those in higher tension (Fig. 5). On the other hand, in the absence of glucose the oxygen uptake under 100 per cent oxygen was about 30 per cent greater than under 5 per cent oxygen. High oxygen tension was toxic to the glucose respiration of *T. vaginalis*, although it simulated the endogeneous respiration. In other words, the increase in oxygen uptake caused by the addition of glucose was completely canceled under 100 per cent oxygen by oxygen toxicity. These facts suggest that the endogeneous respiration is operated by a mechanism which is different from that in glucose respiration. The high tension caused the rapidly increasing inhibition of glucose respiration. This effect might possibly be attributed to the accumulation of such a toxic product as hydrogen peroxide.

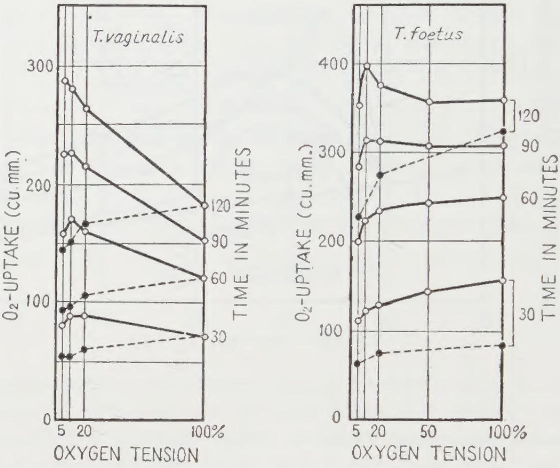


FIG. 5. Effect of oxygen tension upon *T. vaginalis* and *T. foetus*.
●------● Endogeneous.
○——○ With 0.02 *M* glucose as substrate.

Catalase Activity of T. vaginalis and T. foetus—In order to ascertain whether oxygen may participate in a reaction producing hydrogen peroxide, the following experiments were conducted. After 60 minutes of the manometric runs for measuring the oxygen uptake under pure oxygen, the reaction mixtures were centrifuged and the amounts of hydrogen peroxide present in the supernatants were measured. As shown in Table IIa, it was ascertained by manometry that a small but definite amount of oxygen was produced by the addition of catalase preparation of rat liver.

TABLE I

Effect in Inhibitors upon Oxygen Uptake by T. vaginalis

Inhibitor	Concentration	Inhibition	Gas phase	Substrate*
	M	*per cent*		
Potassium cyanide**	0.005	0	Air	Maltose
Sodium azide	0.1	28	//	//
	0.01	0	//	//
	0.001	24	$5\%O_2$-$95\%N_2$	Glucose
	0.001	+22***	100% O_2	//
Malonate	0.1	0	Air	Maltose
Sodium fluoride	0.02	96	//	//
	0.004	38	//	//
Iodoacetate	0.0001	85	//	//
	0.00001	28	//	//
Arsenite	0.005	75	//	//
	0.001	29	//	//
PCMB	0.00005	78	//	Glucose
Arsenate	0.01	0	//	Maltose
Hydrogen peroxide	0.0006	67	//	Glucose
	0.00006	6	//	//

* The final concentration of substrate was 0.02 *M*. Inhibitors were used after neutralization, if necessary. The inhibition per cent was calculated from O_2-uptake for 40–60 minutes.

** Used alkali-cyanid mixture in centre well.

*** Activated.

Then, the influence of hydrogen peroxide upon the respiration was studied. As shown in Table I, hydrogen peroxide caused 75 per cent inhibition in its final concentration of 0.0006 *M*. Thus it was indicated that this concentration was comparable with that accumulated in the former case. The catalase activities of parasite preparations were measured (Table IIb). *T. vaginalis* suspension had no catalase activity at all. The parasite extract obtained by the freezing-thawing method showed extremely weak catalase activity. Comparing it with the Q_{kat} values of various cells (*4*), it would be concluded that the parasite possessed no catalase activity. The accumulation of hydrogen peroxide might be the possible reason for oxygen toxicity. On the other hand, *T foetus* clearly showed the passable catalase activity Table IIb).

TABLE II

Accumulation of Hydrogen Peroxide in Manometric Reaction Mixtures and Catalase Activity of T. vaginalis and T. foetus

IIa. *Accumulation of Hydrogen Peroxide**

System	O_2 produced (*cu. mm. per 15 minutes*)	
Supernatants+catalase solution	13.4 16.0 11.2	mean 13.5
Supernatant+heated catalase solution**	0	

IIb. *Catalase Activity of T. vaginalis and T. foetus****

System	O_2 produced in 30 minutes	Dry weight	Q_{kat}
T. vaginalis	*cu. mm.*	*mg.*	
Suspension	0	3.0	0
Minced extract	12.3	1.4	9
Minced extract+azide****	1.7	1.4	—
T. foetus			
Suspension	104	0.13	800
Suspension-azide****	1.2	0.13	—
Minced extract	36.4	0.02	1600
Basal medium for *T. vaginalis*	48.2	38.5	1
〃 〃 +azide****	2.4	38.5	—
Heated basal medium**	0	—	—

* The system contained 1.0 ml. of supernatant, 0.8 ml. of 0.05 *M* phosphate buffer at pH 7.0 and 0.2 ml. of catalase solution (tipped in from side arm). 20% KOH in centre well. Temperature, 20°.

** Boiled at 100° for 5 minutes.

*** The system contained 1.0 ml. of sample, 0.2 ml. of 0.1 *M* phosphate buffer at pH 7.0, 0.6 ml. of NaCl solution (1.2 percent) and 0.2 ml. of 0.1 *M* H_2O_2 Temperature,

**** The final concentration of 0.01 *M*.

As shown in Table I, azide had no effect on the respiration in air. However, azide inhibited the glucose respiration in 5 per cent oxygen, but rather stimulated it in 100 per cent oxygen. This strange behaviour of azide could not be explained from the standpoint of the inhibition of catalase.

Anaerobic Gas Evolution by T. vaginalis—It was previously reported (*1*) that *T. foetus* produced mainly carbon dioxide and hydrogen in the anaerobic carbohydrate utilization. In the following experiments there

TABLE III

The Anaerobic Gas Evolution by T. vaginalis

IIIa. *Effect of Some Substrates upon tth Anaerobic Gas Evolution*

Substrate**	Total gas evolution (A)		Gas other than barbon dioxide (B)		Carbon dioxide (A)—(B)	
	Found	Substracted endog.	Found	Substracted endog.	Found	Substracted endog.
	cu. mm.	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
None (endog.)	169		65		104	
Glucose	380	211	178	113	202	98
Maltose	266	97	—	—	—	—
Pyruvate	444	275	131	66	313	209
Lactate	191	22	84	19	107	3

IIIb. *Effect of pH upon Evolved Gas other than Carbon Dioxide****

pH	4.2	5.4	6.2	7.8	8.4
Gas evolution (*cu. mm.*)	14	74	104	46	30

IIIc. *Effect of -SH Inhabitors upon the Anaerobic Total Gas Evolution*

Substrate**	Inhibition per cent	
	0.00001 *M* Iodoacetate	0.0001 *M* PCMB
Glucose	73 *per cent*	93 *per cent*
Pyruvate	15	—

* The values are given as cubic millimeters per hour. Gas phase, N_2. Temperature, 37°.

** The final concentration of 0.02 *M*.

*** With 0.02 *M* glucose as substrate.

was no profitable method to meausre manometrically hydrogen evolution. So the total gas evolution and the evolution of gas other than carbon dioxide were determined by using a parir of Warburg flasks with or without KOH in the center well. Thus, the evolution of carbon dioxide could be indirectly determined, and the evolved gas other than carbon dioxide was thought to be mainly composed of hydrogen.

First of all, the effect of various substrates on the anaerobic gas evolution was studied (Table IIIa). The addition of glucose, maltose and pyruvate to the suspension increased the evolution of both carbon-dioxide and the total gas. Lactate and malate increased slightly the total gas evolution. Acetate, formate succinate, fumarate, citrate, glutamate and gluconate did not increase it, but some of them even showed slight inhibitions. The evolution of gas other than carbon dioxide was maximal at pH 6.2 (Table IIIb).

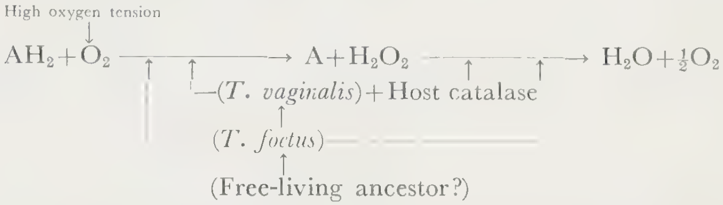
Iodoacetate and PCMB exerted strongly the inhibitory effects on the total gas evolution with glucose, but a much weaker inhibition was observed when pyruvate was used as substrate (Table III c).

DISCUSSIONS

Comparative Aspects of the Carbohydrate Metabolism of Trichomonads—As far as the authors' studies were concerned, the metabolism of *T. vaginalis* seemed to have many similarities to that of *T. foetus*. Especially, both trichomonads were very sensitive to -SH reagents, but quite insensitive to cyanide and malonate. There existed, however, some differences in the following points.

The optimal pH of oxygen uptake by *T. vaginalis* (pH 5.6) was more acidic than that of *T. foetus* (pH 7.2). In spite of the inability of *T. foetus* to oxidize lactate and pyruvate, both of them were somewhat utilized by *T. vaginalis*. On adding pyruvate to the suspension of *T. vaginalis*, there occurred an immediate but short depression of oxygen uptake, followed by a subsequent stimulation (Fig. 4). It is reasonable to think that this depression is caused not by the inhibition of oxygen uptake, but by the evolution of some gas that is not absorbed KOH. Under anaerobic condition pyruvate stimulated the total gas evolution of *T. vaginalis* to the same extent as glucose, although a little in the case of *T. foetus*. In the presence of glucose the respiration of *T. vaginalis* was more susceptible to oxygen tension than that of *T. foetus*. The sensitivity of *T. vaginalis* to oxygen tension is quite similar to that of *T. hepatica* reported by Willems *et al.* (*5*). In point of the catalase activity, there is a noticeable distinction between *T. vaginalis* and *T. foetus*. In contrast to *T. foetus*, *T. vaginalis* has no catalase activity at all.

Oxygen Toxicity—The inhibitory effects of high oxygen tensions on the respiration of several parasites have been reported by many other investigators (*6*, *7*, *8*,) and reviewed by Moulder (*9*). It is, however, noteworthy that the oxygen uptake of *T. vaginalis* is inhibited even in the air and that the inhibition does not occur in the absence of glucose. It was demonstrated that the respiration accompanied by the accumulation of hydrogen peroxide and that the parasite possessed no appreciable catalase activity. Considering these facts, it is likely that hydrogen peroxide is produced at a faster rate in higher oxygen tensions by a reaction obeying the law of mass action:



T. vaginalis has its natural habitat in the vagina of mammalians where there is low oxygen tension. Therefore, in the course of the evolution from free-living to parasitic existence, *T. vaginalis* must be in inclination to adapt itself to the anaerobic mode of life. Many cultural experiments indicated that *T. vaginalis* required serum as well as cysteine (or thioglycollate) for its growth. Cysteine is necessary for lowering the redox potential of the medium, because it has been found that aerobic fermentation does not occur in its absence. The accumulation of hydrogen peroxide is not suitable for the anaerobiosis, for its presence raises up the potential. In consideration of these facts, serum is not only nutritionally necessary but also favorable in decomposing hydrogen peroxide by its catalase activity so that the parasite may grow *in vitro*. On the contrary, *T. vaginalis* in parasitic existence can utilize catalase which is rich in host tissues, even if it produces hydrogen peroxide. If so, it would not be too much to say that the parasite has lose its own catalase activity owing to its dependence on the host catalase. The adaptation to a parasitic existence is usually thought of as occurring through the progressive loss of enzymes which are no longer essential for survival in the new environment (*10*). *T. vaginalis* is known to be nutritionarilly more exacting as well as more anaerobiotic than *T. foetus*. All these facts lead us to propose the following assumption that in the course of the evolution to parasitism *T. foetus* may be an intermediary type occurring between *T. vaginalis* and such a free-living ancestor as *T. keilini*.

SUMMARY

Using the suspension of *Trichomonas vaginalis*, a parasitic protozoon, the carbohydrate metablism were studied manometrically both under aerobic and anaerobic conditions.

1. The effects of pH, the concentration of NaCl and oxygen tension upon the respiration were preliminarily investigated.

2. Of 11 substrates tested, only glucose, maltose and lactate were oxidized by *T. vaginalis*.

3. The respiration was highly sensitive to -SH inhibitors (*p*-chloromercuribenzoate, monoiodoacetate and arsenite) and also susceptible to fluoride. Cyanide, azide, malonate and arsenate had no inhibitory effect.

4. High oxygen tension inhibited almost completely the increased oxygen uptake in the presence of glucose, while it increased autorespiration. It was concluded that the inhibitory effect of oxygen depended mainly on the accumulation of hydrogen peroxide formed during glucose oxidation. In contrast to *T. foetus* ($Q_{kat}=800$), *T. vaginalis* had no catalase activity ($Q_{kat}=0$).

5. Under anaerobic condition, *T. vaginalis* evolved not only carbon dioxide, but some other gases. The gas evolution increased by the addition of glucose or pyruvate, but rather decreased by -SH inhibitors.

A glance was given upon the comparative biochemistry of trichomonads, and the results obtained were also discussed from the view point of the evolutional adaptation from "free-living" to "parasitic" existence of these organisms.

RESUMO

Uzant la suspendaĵon de parazita protozoo, Trichomonas vaginalis, ni esploris manometre la karbohidrato-metabolon sub ambaŭ aerobaj kaj anaerobaj condiĉoj. Mi unue determinis la plej favoran Kondiĉon por mezuri la spiradon. Nome, ni studis pri efikojn de pH, NaCl-denseco kaj O_2-tensiono sur ĝi.

1. Glukozo, maltozo, piruvato kaj laktato estis utiligataj en la spiradon sed aliflanke, acetato, formato, citrato, sukocenato, glutaminato, alanino kaj glukonato tute ne havis kapablojn pliigi ĝin.

2. -SH Malhelpantoj, nome p-klorohidrargobenzoato, arsenito kaj monojodacetato forte malhelpis la spiradon eĉ en iliaj maldencaj densecoj. Cianido, azido kaj malonato ne montris malhelpajn agojn al ĝi.
3. La altaj oksigeno-tensionoj malhelpis la enspiron kun glukozo, kvankam ĝi pliigis sensubstratan oksigen-enspiron. Sajnis ke la malhelpado estis kaŭzita de H_2O_2 akumulita en la daŭro de spirado. La protozoo ne havis katalazo-aktivecon, dume T. foetus posedis ĝin.
4. Sub anaeroba kondiĉo T. vaginalis produkis karbonan dioksidon kaj aliajn gasojn. Tiu ĉi gas-produko estis stimulata de aldoni glukozon aŭ privaton kaj malhelpita de -SH malhelpantoj.

The authors are deeply indebted to Mr. Y. Hamada, H. Huzitani and K. Okamoto of this laboratory for the cultivation of the parasites and to Dr. I. Tyuma of the Nara Medical College for his generous gift of PCMB. The authors express our grateful thanks to Dr. S. Kuwada, Director of this laboratory for his permission to this investigation and publication and to Prof. F. Egami for his encouragements. The authors' thanks are also due to Assistant Prof. S. Inoki of Institute for Microbial Diseases, Osaka University.

REFERENCES

(*1*) Suzuoki-Z. and Suzuoki-T., *J. Biochem.* **38**, 237 (1951)

(*2*) Ninomiya, H. and Suzuki-Z., *J. Jap. Biochem. Soc.* **23**, 116 (1951)

(*3*) Umbreit, N. *et al.*, *Manometric Techniques and Tissue Metabolism* (1949)

(*4*) Fujita, A. and Kodama, T., *Biochem. Z.* **232**, 20 (1931)

(*5*) Willems, R., Massart, L. and Peeters, G., *Naturwiss*, **30**, 169 (1942)

(*6*) Silverman, M., Ceithamal, J. Taliaferro, L.G. and Evans, E.A., *J. Infect. Dis.* **75**, 212 (1944)

(*7*) Moulder, J.W., *J. Infect. Dis.* **83**, 33 (1948)

(*8*) Laser, H., *Biochem. J.* **38**, 333 (1944)

(*9*) Moulder, J.W., *J. Parasitol.* **36**, 193 (1950)

(*10*) Moulder, J.W., *Ann. Rev. Microbiol.* **3**, 101 (1948)

The Journal of Biochemistry, Vol. 39, No. 4

PAPER CHROMATOGRAPHY OF SOME KETOBILE ACIDS

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(Received for publication, February 28, 1952)

Since the report on the use of paper chromatography for the detection of amino acids was published by Martin and others (*1*), this new method has been increasingly used by many researchers in the field of steroid chemistry (*2—11*).

In applying the method to the analysis of bile acids, two points should be borne in mind. (a) The extremely low solubility of bile acids in water, and (b) the absence of a suitable, noncorrosive coloring reagents for the acid detection. These two make the work very difficult. In order to eliminate these difficulties, attempts were made to prepare a *p*-phenyl diazobenzene carboxylic esters, or to use a nonhydrous partition chromatography or a pregnated paper, but it was unsuccessful as far as the simplification of the method and good separation were concerned. Therefore, Zaffaroni's method of developing ketosteroids as the Girard T hydrazone was applied to ketobile acids. This gave the conclusions that ketobile acids with different number of ketone groups could be separated by this method, and that the ketone group at C_3 did not form a stable hydrazone with Girard T.

EXPERIMENTAL

Material—

(a) The sample used: The methyl esters used in this study were freshly prepared by the usual methods. The esters were used because they are easily crystallized and also have definite physical constants. Furthermore, the esterification of bile acids is so simple that it seemed to offer no inconveniences in applying the present method. The ketone group at C_3 was confirmed by oxime formation and the Zimmermann reaction.

(b) Filter paper: Toyo Roshi No. 131.

(c) Reagent: Commercial Girard T hydrochloride, recrystallized from dehydrated alcohol and stored in a desiccator.

(d) Solvent used in Hydrazone Formation: Dehydrated methanol, obtained in the usual manner, containing 10 per cent by volume of glacial acetic acid.

(e) Developing solvent: Freshly distilled *n*-butanol (b.p., 114-118°) and *iso*-propanol (b.p., 81–85°).

(f) Coloring Reagents: A mixture of 5 ml. of 5 percent hydrochloric acid solution of platine chloride, 45 ml. of 10 per cent aqueous solution of potassium iodide and 100 ml. of distilled water, stored in a dark place.

Hydrazone Formation with Girard T—In order to facilitate the handling of a small amount of substances, a simplified method was used. A 1–2 mg.-portion of the sample was measured into a capillary, 1–2 mm. in diameter and 10–12 cm. in length, followed by the addition of twice its weight of Girard T hydrochloride, and 80–100 mg. of the solvent and sealed. After shaking thoroughly to mix the contents, it was allowed to stand overnight at 40°.

Chromatography—For individual runs, filter paper strips of 2×40 cm. were used. For comparative runs of several samples, a sheet of 30×40 cm. were used.

The capillary reaction tube used was opened at one end, and the opposite empty end of the tube was lightly grasped by which a desired amount of the mixture was placed on the filter paper.

The amount of the reaction mixture to be placed on the filter paper is usually 0.003 ml., but this amount should vary according to the nature of the derivatives used. When a pure substance is used, the degree of coloration will depend on the number of the hydrazone residue bound to the substance so that the number of Girard T should also be taken into consideration.

In the case of a mixture of several keto acids many spots will be found and the degree of coloration of these spots increases with the decreasing value of R_f, *i.e.*, with the increasing number of the hydrazone residue bound to the substance. If a good separation of chromatogram in the large R_f value range is desired, a comparatively large amount of the reaction mixture should be used. If a separation in the smaller R_f value range is preferable, a smaller amount of the mixture is to be applied.

The filter paper spotted with the reaction mixture is developed by the ordinary method with a mixture of *n*-butanol, *iso*-propanol and water in the ratio of 6:2:1. When the front of the solvent has reached

20–30 cm. from the original spot, the paper is taken out and the front marked. This is then dried at a room temperature, sprayed with the coloring reagent, and the excess of the reagent carefully removed by running water. While drying, the hydrazone of ketobile acids will appear as purple spots, and the excess of Girard T as black spots, the rest being discolored.

RESULTS AND DISCUSSION

The R_f values of 14 kinds of the hydrazones of ketobile acid esters are given in Table I. Each value is the mean value of several runs repeated at a room temperature during May and June. The variability of the R_f values was 5 per cent which was assumed to be due to the variation in temperature, concentration, and the amount used.

When, as was usually the case, several samples were chromatographed on one sheet by the ascending method, there was no change in the order of the R_f values of each sample, although a slight difference in R_f value of individual substance was found in consecutive runs.

TABLE I

Substance	Substituents			
	C_3	C_7	C_{12}	R_f
Me 3-Ketocholanate	C=O	H	H	no spot
Me 3-Keto-12-hydroxycholanate	C=O	H	OH	,,
Me 3-Keto-12-acetoxycholanate	C=O	H	AcO	,,
Me 3-Keto-7, 12-dihydroxycholanate	C=O	OH	OH	,,
Me 3-Keto-7, 12-diacetoxycholanate	C=O	AcO	AcO	,,
Me 12-Ketocholanate	H	H	C=O	0.83
Me 12-Keto-3-acetoxycholanate	AcO	H	C=O	0.76
Me 12-Keto-3-hydroxycholanate	OH	H	C=O	0.74
Me 3, 12-Diketocholanate	C=O	H	C=O	0.70
Me 7-Keto-3, 12-dihydroxycholanate	OH	C=O	OH	0.58
Me 12-Hydroxy-3, 7-diketocholanate	C=O	C=O	OH	0.53
Me 7, 12-Diketocholanate	H	C=O	C=O	0.42
Me 3-Hydroxy-7, 12-diketocholanate	OH	C=O	C=O	0.23
Me 3, 7, 12-Triketocholanate	C=O	C=O	C=O	0.09

From these results obtained, the following conclusions are drawn which are totally analogous to the experimental results obtained by Zaffaroni with other steroids (*2*):

(a) The R_f values of the hydrazones of ketobile acid esters are predominently dependent upon the number of the hydrazone residue bound to the acid molecule, and to a lesser degree by the other polar residues.

(b) Ketobile acids possessing a ketone group in the 3-position of the saturated A ring are either totally undetected (monoketo compounds) or behave like substances that have one less ketone groups (compounds with more than one ketone group). *cf.*, Methyl 3-ketocholanate, methyl 3, 12-diketocholanate.

It follows, therefore, that the effect of unbonded C_3-ketone group must also be taken into consideration together with the effect of other polar groups against the R_f value.

(c) The R_f values generally decrease by the effect of polar groups, the order of the strength of such an effect being C_3-ketone, hydroxyl and acetoxyl group.

For example, the R_f values of the compounds possessing two bound Girard T hydrazone residues decrease in the order of methyl 7, 12-diketocholanate, methyl 7, 12-diketo-3-hydroxycholanate and methyl 3, 7, 12-triketocholanate.

From the results hereby obtained, it can be concluded that this method may be used for obtaining a clue to the determination of structure of bile acids. Ketobile acids may be used *perse*, while others should be used after being oxidized to keto acids.

Further studies are in progress with ketobile acids other than those used for the present series of experiments.

SUMMARY

Zaffaroni's chromatographic method of developing Girard T hydrazone of ketosteroids was applied to ketobile acids, and similar results were obtained, confirming its applicability in this field.

1. After hydrazone formation, 14 kinds of ketobile acids synthetically prepared were chromatographically developed by *n*-butanol : *iso*-propanol : water (6 : 2 : 1) mixture by which separation was effected.

2. By this method, the ketobile acids possessing a ketone group in the 3-position are either totally undetected or they behave like the substances possessing one ketone group less than they actually have.

3. The effect of other polar groups than ketone groups, was found to be governed by a certain rule.

REFERENCES

(*1*) Consden, R., Gordon, A.H., and Martin, A.J.P., *Biochem. J.*, **38**, 224 (1944)
(*2*) Zaffaroni, A., Burton, R.B., and Keutman, E.H., *J. Biol. Chem.*, **177**, 109 (1949)
(*3*) Zaffaroni, A., Burton, R.B., and Keutman, E.H., *Science*, **111**, 6 (1950)
(*4*) Boscott, R.T., *Nature*, **159**, 342 (1947)
(*5*) Boldimgh, J., *Experientia*, **4**, 270 (1948)
(*6*) Howard, G.A., and Martin, A.J.P., *Biochem. J.*, **46**, 532 (1950)
(*7*) Partridge, S.M., and Swain, T., *Nature*, **166**, 272 (1950)
(*8*) Kritchevsky, D., and Calvin, M., *J. Am. Chem. Soc.*, **72**, 4330 (1950)
(*9*) Bush, I.E., *Nature*, **166**, 445 (1950)
(*10*) McMahon, J.M., Davis, R.B., and Kalnitsky, G., *Proc. Soc. Exp. Biol. Med.*, **75**, 799 (1950)
(*11*) Kritchevsky, T.H., and Tiselius, A., *Science*, **114**, 299 (1951)

The Journal of Biochemistry, Vol. 39, No. 4.

ON THE OXIDATION OF FATTY ACIDS BY PURPLE BACTERIA. II. THE FATE OF ACETATE AND PROPIONATE IN RESPIRATION AND PHOTOSYNTHESIS

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(Received for publication, April 9, 1952)

One of the characteristics of photosynthesizing purple bacteria is their ability of using various fatty acids as hydrogen-donating substrates both in repsiration and photosynthesis, the ultimate hydrogen acceptor being oxygen in the former and carbon dioxide in the latter. Details of the mechanism involved in these oxidative processes as well as the question as to whether each fatty acid is oxidized by a common pathway or in different manners in respiration and photosynthesis have not yet been worked out. Using a non-sulfur purple bacterium *Rhodobacillus palustris* we have previously shown that various fatty acids are oxidized in the dark at different velocities and with the consumption of varying amounts of oxygen per molecule of the acids used, and that these values increased in fairly simple quantitative relationships with the increase of the number of carbon atoms in the acid molecule. The present experiments were undertaken with the view to pursue a similar comparative study of various fatty acids as substrates of photosynthesis as well as to make clear the fates of simple fatty acids, acetic and propionic, in respiration and photosynthesis by the same organism.

METHODS

The bacterium was cultured as previously described (*1*). For photosynthetic experiments freshly harvested bacteria were used, while the experiments of respiration were performed with washed bacterial suspension which had been aerated for three days in order to nimimize the endogeneous respiration. Such a treatment did not affect the activity of cells of using fatty acids as substrates for respiration, but was found impair profoundly the photosynthetic activity of the bacteria. Gas exchange was measured manometrically at 25°. The composition of the reaction media used was as follows:

Main chamber: 2.0 ml. of phosphate buffer ($M/5$, pH 7.0), 2.0 ml. of bacterial suspension (about 200 mg. dry weight), 5.0 ml. of water.

Side bulb: 0.5 ml. of the solution of Na-salts of fatty acids.

Center well: 0.5 ml. of 10% KOH solution (in respiration) or a small amount of palladium asbestos and yellow phosphorus (in photosynthesis) (*2*).

Gas phase: Air (in respiration), or 95% pure N_2 plus 5% CO_2 (in photosynthesis).

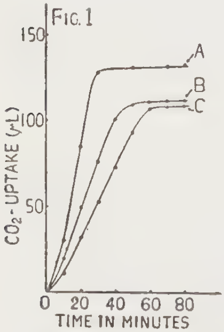
Test was made with ten normal saturated fatty acids, with the number of carbon atom ranging from 2 to 12.

The light source used in photosynthetic experiments was 1000 watt incandescent lamp, the intensity at the bottom of the reaction vessel being more than 15,000 lux which has been found to be saturating for the photosynthesis by our bacterium. The technique employed in the photosynthesis by our bacterium. The technique employed in the determination of oxidation products from acetate and propionate will be described under the individual experiments.

RESULTS

Velocity of Oxidation of Various Fatty Acids in Photosynthesis and the Total Amount of CO_2-Uptake per Molecule of Acids Used—In the presence of fatty acids, the photosynthetic CO_2-uptake proceeded. after an induction period lasting about 5–10 minutes, with a steady rate for a certain period until at a certain stage the velocity suddenly decreased to the value of endogeneous photosynthesis (see Fig. 1). The point of inflection of the curve may be regarded as the time of exhaustion of the acid and the abruptness of the inflection indicates that the acids are used with fairly small Michaelis' constants. The situation is quite similar to those found previously in the experiment of respiration. For each fatty acid the steady rates of CO_2-uptake was found to be constant in a wide range of acid concentration, and this rate increased with the number of carbon atom in the acid molecule up to C_9, and then gradually decreased till it became zero at C_{17}—C_{18}. The increase of the rate with the number of carbon atom

Fig. 1



A: Heptoate, $\frac{1}{2}$ mM
B: Propionate, 1 mM
C: Acetate, 2 mM

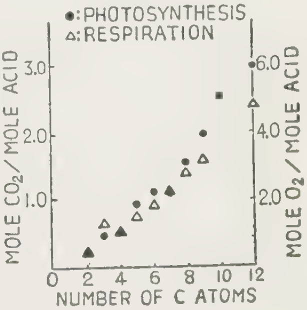
has also been observed in the case of respiration, which, however, showed continued increase in rate up to C_{10}—C_{12}. With $M/2000$ octanoate, for example, which caused the depression of photosynthesis to a value of endogeneous photosynthesis, a fairly strong O_2-uptake has been observed in the dark.* The break of parallelism between respiration and photosynthesis at higher fatty acid concentrations may be interpreted as being due to the higher sensitivity of photosynthetic process to the inhibitory effect of those acids.

The CO_2-uptake to be attributed to the oxidation of the added fatty acid was calculated from the total CO_2 consumption by subtracting the blank CO_2-uptake and adding the CO_2-ouptut in the dark (fermentation). For each fatty acid this net uptake was found to be proportional to the concentration of the acid, and independent of the age as well as the amount of bacteria used. The ratio: (net CO_2-uptake in mole)/(fatty acids used in mole) increased with the number of the carbon atom in the acid molecule (see Table I), a fact which is also in line with our previous observation that in the dark the O_2-uptake per molecule of acid used increased with the length of acid molecule. An analogous, though not quantitatively the same result have been obtained by Gaffron in his photosynthetic experiments using *Athiorhodaceae* (*3*). In Fig. 2 are compared the data obtained in the present experiment with those of the respiration reported in the previous paper.

TABLE I

Number of carbon atoms in fatty acid	CO_2-uptake in mole/acid used in mole
2	0.24
3	0.49
4	0.54
5	0.95
6	1.10
7	1.12
8	1.54
9	1.97
10	2.53
12	3.02

FIG. 2



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* When, however, the concentration of octanoate was lowered to$M/5000$ an appreciable CO_2-uptake occurred in photosynthesis.

Qualitiative Analysis of the Oxidation Products from Acetate and Propionate in Respiration and Photosynthesis—In order to determine the products formed from acetate and propionate in respiration and photosynthesis the following experiments were carried out.

Paralled with a run in the manometric experiment, a larger quantity of the same experimental mixture was shaken under the same condition. The reaction was stopped when the manometric experiment indicated that three quarters of the substrate initially added were used up. After centrifuging off the bacterial cells, the solution and the washings of cells were united, made slightly alkaline with NaOH solution and condensed by vacuum destillation. The condensed fluid was acidified with N-H_2SO_4 and extracted with ether for 12 hours. The ether extract was left standing overnight with dried sodium sulfate, the solvent was evaporated on a water-bath and the unevaporated rest was used for analysis. It should be noted that a preliminary experiment using 0.9% NaCl solution (adjusted to pH 7.0 with diluted NaOH solution) instead of phsophate buffer showed that both in respiration and photosynthesis no change in pH occurred during the incubation, a fact indicating that the number of carboxylic group did not change during the incubation.

(a) *Acetate as Substrate*: The residue of the reaction mixture was extracted with ether and the extract was tested by the technique of paper partition chromatogram, using a mixutre of water-saturated butanol, benzene and acetic acid (4 : 2 : 1) as a developer. After the development the sheet was dried to remove the developer, and then exposed to air for several hours for the complete removal of the volatile acid (acetic acid). Phenol red (in 95 per cent alcohol) was sprayed on the sheet, the latter was then dried quickly in a drying cabinet and the position of the yellow spots on the rose colored sheet was determined. When the pots were diffused owing to some impurities in the ether extract, those portions were cut off, reextracted with ether using a reflux cooler and the extract was subjected once more to the same procedure as above. The position of the spots observed are given in Table II, in which also the data obtained with succinate and fumarate are presented for comparison. As may be seen, the product of photosynthesis gave a spot resembling that of succinate, whereas the product of respiration showed two spots situated close to those of fumarate and succinate.

With the extracts obtained directly from the condensed reaction media as well as those reextracted from chromatogram, various qualitiave tests were made for the substances that were expected as possible pro-

TABLE II

Rf-Values of Substances Contained in the Ether Extracts, as They are Compared with Those of Succinate and Fumarate

Substances	Respiration		Photosynthesis
Ether extracts	0.53	0.61	0.54
Succinate	0.54		
Famarate		0.63	

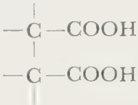
ducts from acetate. Entirely negative results were obtained for glycolic,* glyoxylic, oxalic and malic acids, whereas the pyrol reaction for succinic acid as well as the fluorescence reaction of  $\begin{array}{l} \quad | \\ -C-COOH \\ \quad | \\ -C-COOH \\ \quad | \end{array}$ with resorcinol (*4*) was found to be distinctly positive. The existence of succinate was further confirmed by the enzymatic method using the succinodehydrogenase isolated from horse heart muscle (see Table III).

TABLE III

Enzymatic Test of Succinate in the Oxidation Product of Acetate by the Thunberg Technique

Experimental conditions	Decolorization time of methylene blue
No substrate	120 *minutes*
With substrate	20
Succinate	8

Phosphate buffer: *M*/5 0.25 ml. Methylene blue: (1 : 5,000) 0.25 ml. Solution of ether extract: 0.1 ml. Succinodehydrogenase: 0.1 ml. Water: 18 ml.

Considering the formation of succinate as well as the data obtained in the paper chromatogram, it is probably safe to conclude that the other substance present in the product of respiration was fumarate, though the direct chemical identification of this substance could not be made owing to the lack of adequate method.

(b) *Propionate as Substrate*: The products were extracted with ether

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* Some molds are known to oxidize acetate *via* glycolic acid.

ether in the same manner as above. The samples obtained in respiratory and photosynthetic experiments were found to give qualitiatively almost the same results. On addition of *p*-hydroxydiphenyl after heating the ether extracts with concentrated H_2SO_4, an intensive violet color appeared, indicating the presence of lactate or pyruvate (*5*). Treatment of the extract with lead tetraacetate gave rise to the formation of acetaldehyde (identified by *p*-hydroxydiphenyl), which may be regarded as a proof of the existence of lactate. Further evidence for the presence of lactate was provided by the formation of characteristic crystalls of Zn-lactate on adding Zn-carbonate to the extracts. Test for pyruvate with Na-nitroprussid as well as with 2,4-dinitrophenyl hydrazine* gave positive results, only in the sample obtained from respiratory experiment. It may be concluded that from propionate only lactate is formed in potosynthesis, while pyruvate and lactate are produced in respiration.

DISCUSSION

It has been shown in the previous work that the oxidation of acetate and propionate in the respiration of our bacterium occurred with the consumption of 1/2 and 1 moles O_2 respectively, per mole of substrate, both without formation of any measurable amount of CO_2. In view of the results obtained in the present study, this finding may be accounted for by the following reactions:

$$\text{Acetate} + \tfrac{1}{2}O_2 \longrightarrow \tfrac{1}{2}\ \text{Fumarate} + H_2O$$

$$\text{Propionate} + O_2 \longrightarrow \text{Pyruvate} + 2\ H_2O$$

The succinate and lactate which were found in the products from acetate and propionate, respectively, are conceivably the intermediates in these oxidative reactions, *viz.*

$$\text{Acetate} \xrightarrow[-\frac{1}{2}H_2O]{+\frac{1}{4}O_2} \tfrac{1}{2}\ \text{Succinate} \xrightarrow[-\frac{1}{2}H_2O]{+\frac{1}{4}O_2} \tfrac{1}{2}\ \text{Fumarate} \qquad \text{Eq.(1)**}$$

$$\text{Propionate} \xrightarrow[-H_2O]{+\frac{1}{2}O_2} \text{(Acrylate)} \xrightarrow{+H_2O} \text{Lactate} \xrightarrow[-H_2O]{+\frac{1}{2}O_2} \text{Pyruvate} \qquad \text{Eq.(2)**}$$

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* The hydrazone formed gave deep wine color on dissolving in sodium carbonate solution (*8*).

** As to the mechanism of oxidation of acetate the commonest pathway occurring in animal and plant tissues as well as in microorganisms seems to be the well-known tricarboxylic acid cycle, though the reaction as is represented by Eq. (1) has recently been shown to occur in a soil bacterium, *Corynebacterium creationvorans* (*7*). If the tricarboxylic acid cycle operates, the oxidation of acetate must be accompanied by the evolution of CO_2, which was not the case in our organism.

*** According to Huennekens *et al.* (*8*), the kidney cyclophorase system seems to oxidize propionate in the manner given by this Eq. (2).

It is not clear why the accumulation of these intermediates did not cause a recognizable decrease in the amount of O_2-uptake per mole of substrates consumed. Unfortunately we could not determine quantitatively the relative amount of these substances in the mixture; presumably the accumulation of the intermediates has been, for some reason, slight in our previous experiments.

In the experiment of photosynthesis, the products derived from acetate and propionate were found to be almost the same as those found in the experiments of respiration. As was mentioned already, the net CO_2-uptake per molecule of fatty acids used in photosynthesis increased with the increase of C-atom in the acid molecule in like manner—though not exactly in the same way—as the net O_2-uptake per molecule of acids used in respiration. Moreover, the minimum saturating concentration of each fatty acid appeared to be approximately equal in photosynthesis and respiration. All these findings strongly suggest that the pathway of fatty acid oxidation by our organism is essentially the same in photosynthesis and respiration. Assuming that the photosynthetic reduction of CO_2 occurs in the manner

$$4H+CO_2 \longrightarrow (CH_2O)+H_2O$$

the acetate and propionate metabolism in photosynthesis may be considered to proceed as follows:

$$\text{Acetate} \longrightarrow \tfrac{1}{2}\ \text{Succinate}+H$$
$$\tfrac{1}{4}CO_2+H \longrightarrow \tfrac{1}{4}\ (CH_2O)+\tfrac{1}{4}H_2O$$
$$\text{Acetate}+\tfrac{1}{4}\ CO_2 \longrightarrow \tfrac{1}{2}\ \text{Succinnate}+\tfrac{1}{4}(CH_2O)+\tfrac{1}{4}\ H_2O \qquad \text{Eq.(3)}$$

$$\text{Propionate}+H_2O \longrightarrow \text{Lactate}+2H$$
$$CO_2+2H \longrightarrow \tfrac{1}{2}(CH_2O)+\tfrac{1}{2}H_2O$$
$$\text{Propionate}+CO_2 \longrightarrow \text{Lactate}+\tfrac{1}{2}(CH_2O+\tfrac{1}{2}H_2O \qquad \text{Eq.(4)}$$

According to Eq. (3) and Eq. (4) the consumption of one mole of acetate and propionate will cause the assimilation of 0.25 and 0.5 moles, respectively, of CO_2, which are in good agreement with the observed values (0.24 and 0.49 moles, respectively).

SUMMARY

1. Using a non-sulfur purple bacterium, *Rhodobacillus palustris*, the availability of various fatty acids as the hydrogen donor for the photosynthetic CO_2 reduction was investigated. The oxidation velocity of each fatty acid, as it is reflected in the rate of CO_2-uptake, was found to be constant up to a very low concentration of the acid, indicating

that the Michaelis' constant of the reactions are appreciably small. The rate at saturating concentration of substrate increased with the number of carbon atom in the acid molecule up to C_9, and then gradully decreased till it became zero at C_{17}—C_{18}. The ratio: (net CO_2-uptake in mole)/(fatty acids used in mole) also increased with the number of the carbon atom in the acid molecule. These findings are essentially similar to those made previously in the comparative study of the availability of various fatty acids as substrate of respiration by the same organism. A distinct difference lies, however, in the fact that the photosynthetic activity of bacterial cells is markedly suppressed by the effect of higher fatty acids which could be readily used as the substrate of respiration.

2. Per Oxidation of one mole each of acetate and propionate, 0.24 and 0.49 moles, respectively, of CO_2 were rebuced photosynthetically. Previous experiments showed that in the respiratory process these acids were oxidized with the consumption of 0.5 and 1.0 moles, respectively, of O_2 and indeed without the formation of CO_2.

3. Attempt was made to identify qualitatively the products formed from acetate and propionate in the process of respiration and photosynthesis. It was found that in respiration succinate, probably accompanied by fumarate was formed from acetate, and lactate and probably pyruvate from propionate. On the other hand, the products formed in photosynthesis were found to be succinate from acetate and lactate from propionate.

4. These data were interpreted as indicating that the pathway of fatty acid oxidation by the organism tested is essentially the same in photosynthesis and respiration, and that acetate and propionate are oxidized according to the following fromulae:

$$\text{Acetate} \xrightarrow{-H} \tfrac{1}{2}\ \text{Succinate} \xrightarrow{-H} \tfrac{1}{2}\ \text{Fumarate}$$

$$\text{Propionate} \xrightarrow{-2H} \text{(Acrylate)} \xrightarrow{+H_2O} \text{Lacatate} \xrightarrow{-2H} \text{Pyruvate}$$

It is my great pleasure to thank Prof. Dr. H. Tamiya a for his kind guidance and encouragement throughout this work. This work was supported by a grant from the Scientific Research Fund of the Ministry of Education which is gratefully acknowledged.

REFERENCES

(1) Tsukamoto, A., *Bot. Mag.* (Tokyo, 62, 159 (1949)

(2) Morita, S., Suzuki, K. and Takashima, S., *J. Biochem.*, **38**, 255

(1951)
(3) Gaffron, H., *Biochem. Z.* **275**, 301 (1935)
(4) Feigl, F., *Qualitative Analyse Tupfelreaktionen*, 405 (1938)
(5) Markus, R., *Arch. Biochem.*, 29, 159 (1950)
(7) Hida, T., *J. Shanghai Sci. Institute*, Sec. IV, Vol. **1**, 201 (1935)
(7) Barron, E.S.G., Ardao, M.I. and Hearon, M., *Arch. Biochem.*, **29**, 130 (1950)
(8) Huennekens, F.M., Mahler, H.R. and Nordmanns, J., *Arch. Biochem.* **30**, 66 (1951)

The Journal of Biochemistry, Vol. 39, No. 4.

BINDING OF DYE IONS BY PLASMA PROTEINS IN DISEASES

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(Received for publication, April 12, 1952)

The author reported in the previous paper (*1*) on the spectrophotometric determination of the binding of dye ions by proteins and discussed the results obtained in connection with the spatial configuration of amino acid residue in protein molecules. It was found that in horse and human serum the serum albumin was the only protein component that formed combination with methyl orange and that the maximum number of sites available for the combination of methyl orange per molecule of serum albumin was the same for both pure albumin solution and albumin in plasma.

It is, therefore, possible to determine albumin concentration and accordingly A/G ratio of serum quite easily by the determination of bound dyes. If such a procedure give values of A/G ratio different from those obtained by other methods, e. g., electrophoresis or salting-out, in sera of patients, a conclusion follows that serum albumin in such patients differs from normal serum albumin in dye binding ability.

From the above view point, the author attempted to calculate A/G or $A/G+\phi$ (ϕ: fibrinogen) ratio of serum or plasma from the amount of bound dyes and compared the results with those obtained by electrophoresis.

EXPERIMENTAL

1. Normal human serum or plasma was kindly afforded by the Institute of Hygene, Tokyo Metropolice, and Nihon Seiyaku Co., Ltd. Serum albumin was crystallized after Kekwick's procedure (*2*) and was used for various fundamental investigations of the procedure.

2. Sera of patients were supplied by the Medical Clinic of Prof. Okinaka, S. and the Institute of Physical Therapy and Internal Medicine, Faculty of Medicine Tokyo University. Serum was dialyzed against $1/20\,M$ phosphate buffer at pH 8.0. for 24 hours to remove various kinds of dialyzable ions which might cause competitive

inhibition of binding of methyl orange as well as to carry out all experiments as the same pH and ionic strength.

3. Details of the spectrophotometric determination of bound methyl orange were reported in the previous papers (*1*), (*3*).

4. Protein concentrations were determined colorimetrically by biuret reaction (*4*), micro-Kjeldahl N analysis, and/or by refractometry with Pulfrich refractometer.

5. Electrophoreses were carried out by the Tiselius apparatus in our laboratory or in the Medical Clinic of Prof. Okinaka, S.

RESULTS

1. Relation between Protein Concentration and the Amount of Bound Dye—Normal human serum was serially diluted with 1/15 *M* phosphate buffer at pH 6.8 so that albumin concentrations of diluted solutions became from 0.03 to 0.6 per cent at the interval of about 0.05 per cent. Proper amounts of the same buffer and methyl orange solutions were further added to make all speciments have the same total dye concentrations. E' (see (*1*)) of specimens were measured and the results were plotted against albumin concentration. Curve A of Fig. 1 shows the result of the above procedure. If the binding of human albumin with methyl orange had followed the simple mass action law as in the case of horse serum albumin (*5*), the result would have been a curve of type B in the same figure which was obtained for horse serum by the analoguous procedure. Curve A of Fig. 1 shows that as the albumin concentration becomes higher than 0.2 per cent, E' becomes smaller. This indicates that the binding of human albumin with methyl orange does not follow the simple law of mass action when albumin concentration becomes higher than 0.2 per cent. Initial part, I, of Curve A must be used to determine albumin concentration. Results reported in the previous paper on the maximum number of sites in human albumin molecule for binding of methyl orange was carried out by the analysis of this part of the curve.

Albumins of horse, bovine, hen, and rabbit sera were treated by the same procedure and the results are also shown in Fig. 1. As is readily seen from the figure, binding of methyl orange by these proteins follows the simple law of mass action.

Results reported hereafter in this paper are all those referred to the part I of the curve of type A for human sera.

2. Results Obtained with the Sera of Patients Suffering from Diseases Other than Hepatic Insufficiency—Calibration curve, I, mentioned in the

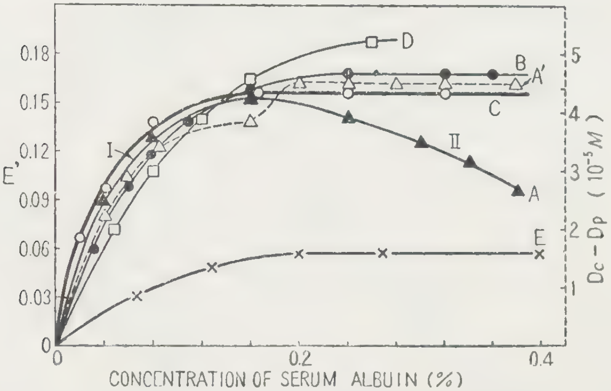

Fig. 1. Relation between serum albumin concentration and amounts of bound methyl orange for various kinds of animals.

A: ▲—▲; A′: △—△ human; B: ○—○ horse; C: ●—● bovine; D: □—□ hen; E: ×—× rabbit.

{continuous lines: results obtained by spectrophotometry.
{broken lines: results obtained by dialysis technique.

Experimental conditions

	Total dye concentration (10^{-5} M)	Temperature
A, A′:	4.55	16°
B :	4.80	19
C :	5.25	12
D :	5.25	12
E :	4.30	12

pH 6.8 in each case.

Dc : dye concentration of outside solution in the control.
Dp : dye concentration of outside solution in the dye-protein systems.

previous section shows remarkable reproducibility within experimental error when the experiments were carried out at the same temperature, and albumin concentration and A/G ratio of normal human sera determined by using this calibration curve is in accordance with the results obtained by electrophoresis (see Fig. 2, solid circle, ●). A/G(D) and A/G(E) are A/G ratios obtained by dye binding and electrophoresis, respectively.

Results obtained for sera of patients without hepatic insufficiency are also plotted in Fig. 2. It is clear from the figure that A/G(D) and A/G(E) for these sera do not differ from each other more than 0.1, and such small differences do not seem to be a serious difficulty for practical purposes.

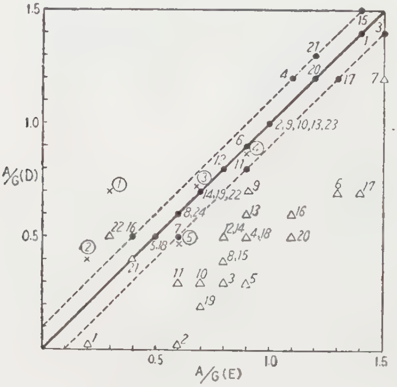

Fig. 2. Comparison of A/G (D) with A/G (E) determined for normal and various pathological sera.

Condition
pH 6.8
Total dye concentration: 5×10^{-5} *M*.
Temperature: 10–11°.
in each case.

No.	Solid circles (●)	Triangles (△)	Crosses (×)
1.	Normal	Hepatic cirrhosis	Nephrosis
2.	,,	,,	(severe) (①)
3.	,,	,,	,,
4.	Pneumonia	,,	(severe) (②)
5.	Pulmonary tuberculosis	,,	,, (③)
6.	Tuberculosis of the kidney	,,	,, (④)
7.	Rheumatoid arthritis	Leucemia	,, (⑤)
8.	,,	,,	
9.	Normal pregnancy	,,	
10.	,,	Icterus	
11.	Basedow's disease	,,	
12.	Diabetus mellitus	,,	
13.	Neurasthenia	Tumor of the mediastinum	
14.	Neoplasm of lymph nodes	Banti's disease	
15.	Addison's disease	Carcinomatous peritonitis (ascites)	
16.	Hyperproteinemia	Serious state of tuberculosis	
17.	Valvular disease	,,	
18.	Gangren of the lung	,,	
19.	,,	Advanced case of gastric cancer	
20.	Typhoid fever (?)	,,	
21.	Acute glomerulonephritis	Icterus (highly icteric sera)	
22.	,,	,,	
23.	Secondary anemia		
24.	Carcinomatous peritonitis		

3. Sera of Patients of Hepatic Insufficiencies—A/G(D) values for sera of patients suffering from primary or secondary hepatic insufficiencies such as hepatic cirrhosis, Icterus simplex, Leukemia, Banti's disease, and serious state of cancer or tuberculosis, are markedly smaller than A/G(E) values (see Fig. 2 triangle, △). Hepatic functions were tested on some of these patients and the lowering of the functions were confirmed in all cases. For several examples of the highly jaundiced sera, on the contrary, A/G(D) values were found to be equal to or even higher than A/G(E) values. Interpretation of these results will be made below.

4. Sera of Nephrotic Patients—Of 5 instances of sera of nephrotic patients investigated, A/G(D) were larger than A/G(E) in two cases and nearly equal to A/G(E) in three, (see Fig. 2 cross ×). A/G(D) and A/G(E) had comparable values when albumin concentration remained above 30 per cent of the total protein, while, when percentage of albumin became less than 20 per cent, sera combined much more dye ions than could be accounted for the concentration of albumin.

DISCUSSION

It seems reasonable to suppose from the above results that albumin concentration determined from the amount of bound dyes for human serum gives correct value except for the case of patients mentioned in sections 3 and 4 of the previous chapter. To use this procedure for clinical and practical applications, detailed investigations on various factors influencing experimental results must further be performed.

Fhe fact that E' decreases with increase in albumin concentration above 0.2 per cent as shown in Fig. 1 indicates that some kind of reactions other than that occuring in lower concentrations of albumin prevail in these range of albumin concentration. Analysis by dialysis technique (*5*) on crystalline human serum albumin yields the result shown by the broken line in Fig. 1, A′. Amounts of bound dye obtained by spectrophotometry (c.f. (3)) and dialysis method agree when albumin concentration is below about 0.2 per cent. At higher concentrations of albumin, dialysis experiment shows that albumin combines dye ions so firmly that they remain little in the outside solution. Wave length of maximum absorption of the inside solution tends to shift to longer wave lengths and become nearly the same as that of the free dye (470 mμ). On the other hand, the maximum absorption of bound dyes for horse and human serum albumin at low concentrations of the latters lies near 450 mμ. That E' becomes smaller at higher albumin concentration is due to the fact that molar extinction coefficients of bound and free dye molecules become nearly the same under these conditions. At any rate, these facts are indicative of the existance of some complex interaction of human serum albumin and methyl orange at higher concentrations of the proteins.

As the causes of marked difference between A/G(D) and A/G(E) values in hepatic diseases, the following possibilities may be considered: (a) Albumin molecules are denatured or cleaved by morbid factors; (b) Albumin molecules synthesized in morbid liver differ in configuration from that synthesized in normal liver; (c) Increased amount of serum bilirubin competes with dye anions in binding with albumin molecules.

With regard to the possibility (a) and (b), the author reported in a previous paper (*1*) that dye binding ability of albumin decreases when it is denatured or partially hydrolyzed by proteolytic enzymes. The decrease in dye binding ability observed in the case of hepatic diseases seems to the author to have relation to the difference in structure of albumin synthesized in normal and morbid livers. In accordance with the results obtained by various methods, such as cephaline-clolesterol flocculation test, thymol turbidity test and Takada-Ara reaction (*6*), present results show clearly the differences in physicochemical properties of serum albumin in these diseases from normal one, although they behave nearly in the same manner electrophoretically.

With regard to the possibility (c) mentioned above, it would be ruled out when bilirubin concentration of serum is nearly in the range of normal value, but in highly jaundiced serum, there is ample evidence that bilirubin combines with serum albumin to give a complex having the same wave length (480 mμ) of maximum absorption as free methyl orange. (Martin, (*7*)). When methyl orange is added to such a serum, free bilirubin increases as a result of competitive binding. Free bilirubin has maximum absorption at shorter wave lengths (430—435 mμ), and the increase in E′ is brought about by both processes of combination of methyl orange and dissociation of bilirubin. This is the possible interpretation of the larger value of A/G(D) than A/G(E) in highly jaundiced sera.

Increase in dye binding ability in nephrotic sera is hardly interpretable. With regard to these points the author is going to perform more detailed investigations.

SUMMARY

Spectrophotometric analysis of the binding of dye by proteins has yielded the following results.

1. Human serum albumin shows two step combination reaction with methyl orange, while horse, bovine, hen, and rabbit serum albumins show only one step reaction.

2. Albumin concentration and therefore the A/G ratio of normal and various pathological sera can be determined fairly accurately by spectrophotometric determination of bound dyes except in the case of hepatic insufficiency and nephrosis.

3. Serum proteins in severe hepatic insufficiency bind less dye ions than can be accounted for on the basis of albumin concentration.

The author expresses his cordial thanks to Prof. K. Kodama and Assist. Prof. H. Yoshikawa for their kind guidance and also to Dr. H. Hirai and Mr. K. Shimao for their helpful advice and valuable discussion throughout the course of these investigations.

The author is greatly indebted to Dr. K. Sakaguchi, Chief of the First Tokyo National Hospital, Dr. K. Miyoshi and Dr. T. Sasaki in Tokyo University Hospital, and Dr. F. Yanagisawa for their kind supply of normal and pathological sera used in this study.

REFERENCES

(*1*) Kusunoki, T., *J. Biochem.*, **39**, 245 (1952)
(*2*) Kekwick, R.A., *Biochem. J.*, **32**, 552 (1938)
(*3*) Shimao, K., *J. Biochem.*, **39**, 241 (1952)
(*4*) Allan, G.G., Charles, J.B., and Maxima, M.D., *J. Biol. Chem.*, **177**, 1751 (1949)
(*5*) Klotz, I.M., Walker, F.M., and Pivan, R.B., *J. Am. Chem. Soc.*, **68**, 1486 (1946)
(*6*) Gutman, A.B., *Adv. in Prot. Chem.*, **4**, 155 (1948)
(*7*) Martin, N.M., *J. Am. Chem. Soc.*, **71**, 1230 (1949)

The Journal of Biochemistry, Vol. 39, No. 4

STUDIES ON THE INTERACTION BETWEEN PYRIDINE-HEMIN AND HYDROGEN PEROXIDE OR OXYGEN.
IV. ON THE FORMATION OF 630-COMPOUND IN THE REACTION SYSTEM OF PYRIDINE-HEMIN—ASCORBIC ACID—HYDROGEN PEROXIDE

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(Received for publication, April 24, 1952)

As reported by several workers, pyridine-hemin can easily be converted by H_2O_2 into 630-compound in the presence of ascorbate (*1*). In the absence of ascorbate, however, the formation of 630-compound is not observed but there arises another new product as described in our previous paper (*2*). The object of the present investigation is to study in detail the former reaction, namely the reaction in the presence of ascorbate.

EXPERIMENTAL METHODS

The experiments were carried out using an absorption cell in the following manner: 3.0 ml. of 1.1×10^{-4} *M* solution of pyridine-hemin in 20 per cent aequous pyridine were placed in the cell of 1 cm. depth, covered with liquid paraffine, and then N_2 gas from a glass capillary was bubbled through the solution to drive out the last trace of O_2 gas dissolved in the solution. 0.1 ml. of ascorbate solution of a definite concentration was added to it and the mixture was stirred gently with a glass rod. 0.2 ml. of H_2O_2 was then added and finally the solution was mixed promptly. The spectral measurements were carried out by the same apparatus as described in the previous paper. The reaction was in completion in 2–3 minutes at room temperature (7–9°). All the spectral measurements were, therefore, undertaken after three minutes. The reaction between H_2O_2 and ascorbic acid without hemin-catalysis was negligibly small as confirmed iodometrically.

RESULTS

Absorption-Concentration Curve of 630-Compound—The 630-compound in its ferric state is characterized by its absorption in the red with its

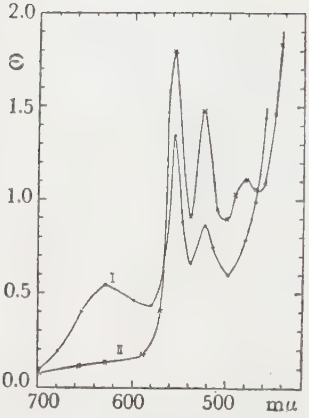


Fig. 1. Absorption spectra of the reaction solution in which the formation of 630-compound is in completion.

$1\times10^{-4}\,M$ pyridine-hemin, $0.125\times10^{-2}\,M$ ascorbic acid, $0.125\times10^{-2}\,M$ H_2O_2. pH 8.6. I. After 3 minutes of the reaction. II. Reduction of I with $Na_2S_2O_4$.

maximum at 630 mμ as shown in Fig. 1 of this paper and in Fig. 2 of the previous paper. In its ferrous state obtained by reduction with $Na_2S_2O_4$, the absorption in the red disappears and there arise instead two absorption maxima in the green at 557 and 525 mμ. These positions of wavelength are in agreement with those of pyridine-hemochrome, while the relative intensity of the ε_{557} to that of ε_{525} at their maxima differs from that of the latter, namely ε_{557} being smaller than ε_{525} in the case of 630-compound.

The following series of experiments with varying concentrations of H_2O_2 were undertaken: 3.0 ml. of $1.1\times10^{-4}\,M$ pyridine-hemin; 0.1 ml. of $3.3\times10^{-1}\,M$ ascorbate; 0.2 ml. of H_2O_2 in varying concentrations. In each experiment, the optical density of 630 mμ was first measured after three minutes and then the solution was immediately reduced with $Na_2S_2O_4$ with subsequent measurement of ε_{557} and ε_{525}. The optical densities thus measured were plotted against the log concentrations of H_2O_2 (Fig. 2). As shown in the figure, the formation of 630-compound increased as the concentration of H_2O_2 increased until ε_{630} reached its maximum limit, 0.8. At this point, the formation of 630-compound reached maximum and pyridine-hemin had been completely converted to form 630-compound in view of the fact that the further addition of H_2O_2 to this solution did not increase the absorption intensity corresponding to 630-compound. From the several experiments repeated, the ratio $\varepsilon_{557}/\varepsilon_{525}$ for the 630-compound in its ferrous state was found to be 0.75 in average. The same ratio, $\varepsilon_{557}/\varepsilon_{525}$, for the pure pyridine-hemochrome was found to be 1.90. These two values of the ratio are respectively the values of the two substances in their pure state. The value of this ratio can, thus, be used as a measure of 630-compound formation or of pyridine-hemin decomposition respectively, but only under the assumption that the 630-compound formation represents exactly the decomposition of pyridine-hemochrome,

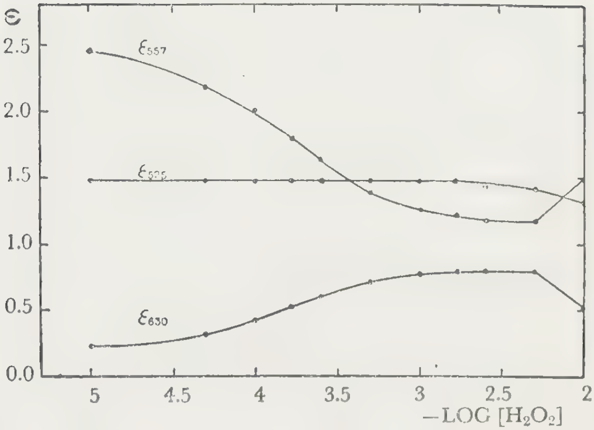


Fig. 2. Relations between the concentrations of H_2O_2 added and the optical densities at each wave length marked in the reaction solutions. pH 8.14. ε_{630}: after 3 minutes of the reaction. ε_{557}, ε_{525}: after solutions were reduced with $Na_2S_2O_4$ immediately after the measurement of ε_{630}.

and this is possible under the carefully selected conditions in which the further decomposition of 630-compound can be avoided. By the use of H_2O_2 solution of exceedingly high concentrations the 630-compound once formed will be subjected to further decomposition, resulting in a decrease of the absorptions characteristic of the compound.

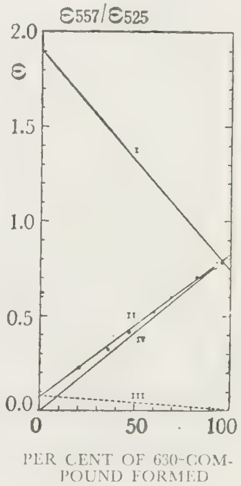


Fig. 3. Procedures to obtain the absorption-concentration curve of 630-compound. (Based on the experimental data in Fig. 2).

I. Nomogram representing the theoretical relationship between the per cent formation of 630-compound and the ratios of $\varepsilon_{557}/\varepsilon_{525}$ of the solutions after they were reduced with $Na_2S_2O_4$. II. ε_{630} of the reaction solutions corresponding to the per cent formation of 630-compound. III. Nomogram representing the contributions of the pyridine-hemochrome fractions remaining unaffected in the solutions to the value of ε_{630} in each per cent formation of 630-compound. IV. Theoretical absorption-concentration curve of ferric 630-compound.

Thus the percentage formation of 630-compound can be determined by a graphical method by the use of the straight line I in Fig. 3.

Now the value ε_{630} of the reaction solution was measured in each experiment and was plotted against the corresponding percentage formation of 630-compound determined from the ratio $\varepsilon_{557}/\varepsilon_{525}$. The relation is shown by line II in Fig. 3. As the value ε_{630} of $\mathrm{Fe}^{++}<^{\mathrm{Pyr}}_{\mathrm{Pyr}}$ in $1.0\times10^{-4}\,M$ is equal to 0.08 when no 630-compound is formed, the plotted points must lie on the straight line crossing 0.80 of ε_{630}, if the reaction proceeds quantitatively without any formation of byproducts. This is in agreement with the experimental results, indicating that the decomposition of $\mathrm{Fe}<^{\mathrm{Pyr}}_{\mathrm{Pyr}}$ corresponds exactly to the formation of 630-compound. A straight line IV in Fig. 3 is thus drawn by subtracting III from II, and this gives the concentration vs. absorption curve of the 630-compound. The theoretical value of ε_{630} of 630-compound, at its complete formation, was determined by the extrapolation as 0.82, the initial concentration of hemin being $1\times10^{-4}\,M$ as in the present experiment. Thus the molar extinction coefficient is given as $\varepsilon_{\mathrm{mol}}=0.82\times10^{4}$.

Formation of 630-Compound as a Function of the Amount of H_2O_2 Added—By the calculation described above, it was possible to determine the formation of 630-compound in each of the given experiments. A more distinct representation of the relationship between the amounts of H_2O_2 added to the system and the resulting formation of 630-compound is shown by Curve I in Fig. 4, which is obtained from Fig. 3 by calculation. Curve I in Fig. 4 includes, however, that portion of 630-compound which was formed by the action of oxygen originally present in a minute amount in the reaction mixture. If

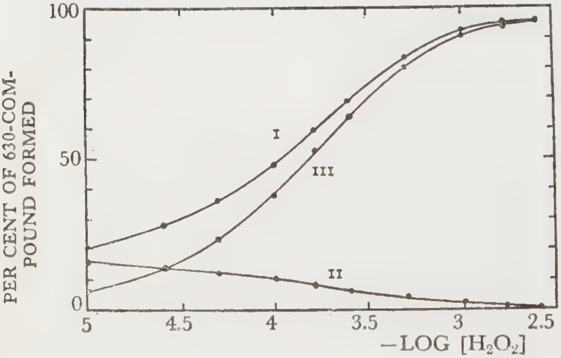


FIG. 4. Relationship between the per cent formation of 630-compound and H_2O_2 added. (Derived from Fig. 2).

I. Calculated from the experimental results as observed. II. Amount of 630-compound, which was formed spontaneously without the effect of H_2O_2 added. III. Theoretical per cent formation of 630-compound in relation to the concentration of H_2O_2 added.

the reaction mixture was kept to stand after the addition of H_2O in place of H_2O_2, 630-compound was actually formed, amounting to 19 per cent in 3 minutes. With the assumption that the amount of 630-compound formed without H_2O_2 was in proportion to the amount of pyridine-hemin which remained unaffected, the amount of 630-compound formed by this process can be calculated by the following equation :

$$x=19\times\frac{100-\mathrm{a}}{100}=0.19\times(100-\mathrm{a})\%$$

where a represents the percentage of 630-compound formed by the action of H_2O_2. x is, however, an approximate value. A more appropriate method for the determination of the value of x is not yet available at the present stage. Curve II in Fig. 4 has, thus, been obtained from Curve I in Fig. 4 by calculation using the above equation. Curve III in Fig. 4 obtained by subtracting the value of II from that of I, gives the true curve of the 630-compound actually formed by H_2O_2 in the system.

Effect of pH—pH of the reaction solution was varied by the addition of varying amounts of NaOH or HCl. The ascorbic acid was neutralized before use with an equivalent amount of NaOH. pH of the reaction mixture was determined by the hydrogen electrode separately with 20 per cent pyridine in place of pyridine-hemin solution and with water in place of ascorbate solution. The results obtained at varying pH are given in Fig. 5. As shown in Fig. 5, the amount of 630-compound

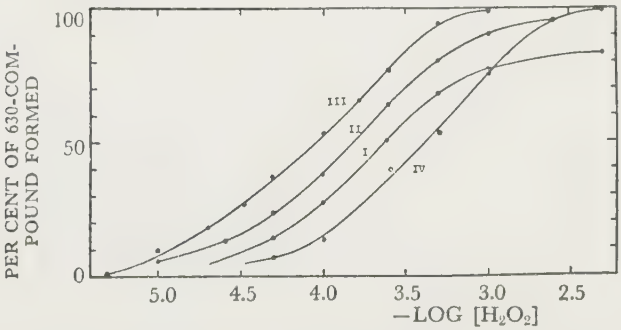


FIG. 5. Effect of pH on the per cent formation of 630-compound as a function of the concentration of H_2O_2 added.

$1\times10^{-4}\,M$ pyridine-hemin, $1\times10^{-2}\,M$ ascorbic acid. pH: I. 6.88, II. 8.14, III. 9.30, IV. 11.96.

formed (which corresponds to the concentration of O_2H_2 added) varied with the change of pH of the medium, but the slope of the curves changed only slightly by the alteration of pH. The optimum pH lies at about 9.3, which nearly corresponds to that of 20% pyridine solution. It is of interest to note that pH 9.3 is also an optimum both for the formation of the 630-compound in the present reaction system and of the verdohemochrome in the reaction system of pyridine-hemin—ascorbic acid—oxygen (2).

Effect of Ascorbate Concentration—The amount of 630-compound formed by the action of H_2O_2 was followed in the reaction system at pH 9.3 with the presence of sodium ascorbate at varying concentrations, the H_2O_2 being added in three different concentrations; (1/2000, 1/6000 and 1/10000 *M*). The results are shown in Fig. 6. As shown in the figure, the amount of 630-compound formed increased as the concentration of the ascorbate increased, until the 630-compound formation reached its maximum. Regardless of the varying concentration of H_2O_2 added, the lowest concentration of the ascorbate sufficient for the maximum formation of 630-compound was always found to be about $0.5 \sim 1.0 \times 10^{-2}$ *M*.

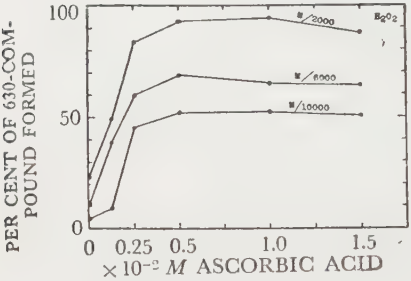


FIG. 6. Relations between the per cent formation of 630-compound and the concentrations of ascorbic acid.

1×10^{-4} *M* pyridine-hemin, pH 9.3.

Catalytic Activities of 630-compound—As Fig. 1 shows, in the reaction solution in which ascorbate and H_2O_2 were kept at equivalent concentrations, a distinct absorption of ferrous hemochrome could be recognized even after 3 minutes beside a marked absorption of 630-compound produced by that time. It is evident, thus, that a considerable amount of ascorbate still remained unaffected. If however, the concentration of ascorbate was, lowered, for example, as in an experiment with 0.125×10^{-2} *M* ascorbate against 0.167×10^{-2} *M* H_2O_2 as their final concentration, no appreciable amount of ferrous hemochrome could be detected after 3 minutes, indicating that it had been wholly converted into ferric hemichrome. Even in this latter case, it has been confirmed that no byproduct has been formed with in 3 minutes. From these facts it is considered that a certain portion of H_2O_2 added has been decomposed by the catalytic action of another hemin derivatives of a special sort arising in the system. As Figs. 3, 4 and 5 show, however, in the presence of ascorbate at sufficiently high concentration, 630-compound thus produced contained no byproduct even when H_2O_2 was added in great excess in proportion to the amount of pyridine-hemin present. Evolution of oxygen bubbles was in no case recognizable in these experiments. It may, therefore, be postulated that a considerable amount of H_2O_2 has been decomposed rather peroxidatically in these experiments.

Another evidence for the catalytic process in this reaction system will be understood from Fig. 7 derived from Curve III, Fig. 5 by calculation. The curve in Fig. 7 indicates the efficiency of H_2O_2 for the formation of 630-compound, expressed by the molar ratio of [630-compound formed]/[H_2O_2 added] as a function of H_2O_2 concentration. According to Fig. 7, the maximum efficiency of H_2O_2 for the formation of 630-compound is found in rather lower range of concentration of H_2O_2 and it decreases as its concentration increases.

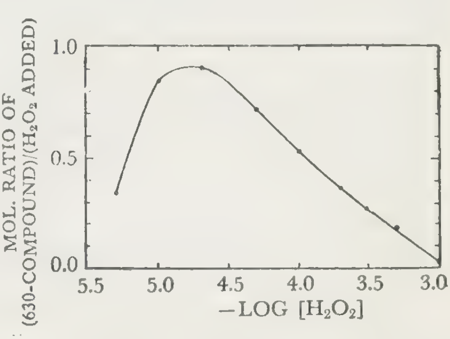


FIG. 7. Change of the efficiency of H_2O_2 for the formation of 630-compound with the alterations of the concentration of H_2O_2.

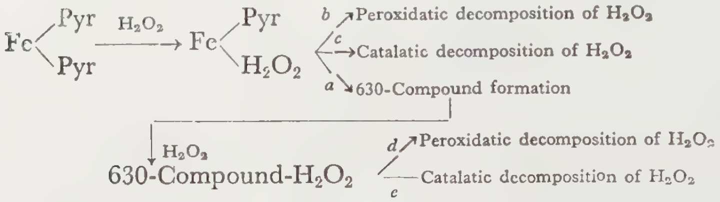
1×10^{-4} *M* pyridine-hemin, 1×10^{-2} *M* ascorbic acid. pH 9.3.

To account for the above fact, it should be pointed out that H_2O_2 in the present system is decomposed through the following three main types of the reaction: (a) 630-compound formation; (b) catalatic and peroxidatic action of the intact pyridine-hemin; and (c) catalatic and peroxidatic actions of 630-compound formed. Similar consideration has been offered with regard to the formation of verdohemochrome in the previous paper. In the present system, however, pyridine-hemin was converted into 630-compound continuously as the reaction proceeded. Moreover, the rate of conversion of pyridine-hemin into 630-compound was increased as the concentration of H_2O_2 increased.

Taking above facts into consideration, it may reasonably be assumed that the catalytic activities of 630-compound, especially the peroxidatic activity, is dominant over any other reaction for the breakdown of H_2O_2 added in the system. The more H_2O_2 is added, the more 630-compound will be formed, and the more H_2O_2 will be broken down peroxidatically by the catalytic action of the increasing 630-compound. The reactions (a), (b) and (c) compete with each other for the decomposition of H_2O_2, so that much more H_2O_2 will be needed for further production of 630-compound in higher ranges of H_2O_2 concentration than in lower ones.

DISCUSSION

As shown in the experimental section, the reactions indicated below are in competition with one another for the breaking-down of H_2O_2 at different stages of the reaction process, which are denoted by a, b, c, d and e, respectively in the following scheme:


Fe
Pyr
Pyr
H₂O₂
Fe
Pyr
H₂O₂
b
Peroxidatic decomposition of H₂O₂
c
Catalatic decomposition of H₂O₂
a
630-Compound formation
H₂O₂
630-Compound-H₂O₂
d
Peroxidatic decomposition of H₂O₂
Catalatic decomposition of H₂O₂
e

It is to be assumed that in each type of these reactions, pyridine-hemin combines with H_2O_2 to form H_2O_2-complex compound as the first step of reaction. Evidence for the formation of H_2O_2-complex has already been established by spectroscopical studies reported in the previous paper (*2*).

It is remarkable that the formation of 630-compound (a in the above scheme) takes place only in the presence of certain reductants such as ascorbic acid. The machanism of the reaction (a) seems to be in close resemblance to that of pyridine-hemin functioning as peroxidase. It is highly probable that the reaction takes place in such a way as follows: under the condition in which pyridine-hemin acts peroxidatically, pyridine-hemin molecule can also be converted into 630-compound through either intramolecular or intermolecular oxidation-reduction, partly undergoing the peroxidatic action of pyridine-hemin. Each of the reactions b, c, d and e, competing with each other for the decomposition of H_2O_2, though different in their mode of action, may have more or less effect on the process of 630-compound formation.

The catalatic and peroxidatic activities of the hemin derivatives can be affected, as is well known, by some such a condition as the pH of the medium or the concentration of hydrogen donor contained. It is of interest to note, however, that as shown in Fig. 5, the curves I∼IV obtained at different pH of the medium run fairly parallel to each other. This phenomenon is as yet unaccountable. From Fig. 5 it is also to be noticed that at relatively high concentration of H_2O_2, the rate of 630-compound formation falls as the pH of the solution decreases. This seems to be mainly due to the increased catalatic activity of pyridine-hemin under decreased pH, a fact which has been reported by Kuhn and Brann (*3*).

At the present stage it is unknown which reaction is the more reponsible one for the formation of 630-compound, the ferrous or the ferric state of pyridine-hemin. Brief consideration has been given provisionally on this problem in the previous paper (*1*). It was suggested therein that some proper redox-potential conditioned by the reaction medium would be necessary for the production of 630-compound, which possibly depends on the rapidity of $Fe^{++} \rightleftharpoons Fe^{+++}$ interchange. In addition to the facts described in the previous papers, it should be pointed out that $0.25 \times 10^{-2}\,M$ of ascorbate is, as shown in Fig. 6, almost sufficient for the completion of 630-compound formation and a further increase in the ascorbate concentration hardly exerts any influence upon it. Thus it is to be considered that the redox-potential of the reaction medium is properly conditioned when ascorbate of this concentration is present.

SUMMARY

1. The final product of pyridine-hemin in the reaction system of pyridine-hemin, ascorbic acid and H_2O_2 is 630-compound. No byproduct formation is observed in the system unless a great excess of H_2O_2 is used.

2. The concentration-absorption curve for the 630-compound was determined; $\varepsilon_{\mathrm{mol}}$ at 630 mμ being equal to 0.82×10^4.

3. The amount of 630-compound formed corresponds to the ammount of H_2O_2 when that of ascorbate is kept constant, while it corresponds to that of ascorbate at a given concentration of H_2O_2.

4. In the formation of 630-compound, the concentration of H_2O_2 plays a greater part than that of ascorbate.

5. The optimum pH for the formation of 630-compound is 9.3.

6. The 630-compound is supposed to be most active as one of the peroxidatic catalysts among the hemin derivatives existing in the system.

The present investigation was supported by the subsidy of the Science Research Fund of the Ministry of Education, for which we express our hearty thanks.

REFERENCES

(*1*) Lemberg, R., Cortis-Jones, B., and Norrie, M., *Biochem. J.*, **32**, 149 (1938); **32**, 171 (1938); Fischer, H., and Libowitzky, H., *Z. physiol. Chem.*, **251**, 198 (1938); **255**, 209 (1938); Libowitzky, H., *Z. physiol. Chem.*, **265**, 191 (1940); Haurowitz, F., *Enzymologia*, **2**, 9 (1937); **4**, 139 (1937); Kaziro, K., and Kikuchi, G., *J. Biochem.*, **38**, 213 (1951); **39**, 63 (1952)

(*2*) Kaziro, K., and Kikuchi, G., *J. Biochem.*, **39**, 193 (1952)

(*3*) Kuhn, R., and Brann, L., *Z. physiol. Chem.*, **168**, 27 (1927)

The Journal of Biochemistry, Vol, 39, No. 4.

ON THE INFLUENCE OF THE ADMINISTRATION OF AMINO ACIDS UPON THE DISTRIBUTION OF PORPHYRIN BODIES IN TISSUES. I. THE INFLUENCE OF THE ADMINISTRATION OF SULPHUR CONTAINING AMINO ACIDS

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(Received for publication, May 8, 1952)

In the preceding experiments (*1*) carried out by one of the authors it has been confirmed that porphyrin and metalloporphyrin (henceforth will be called porphyrin bodies collectively) are constituents of all cells playing important roles as the agons of respiratory enzymes which function in the region in contact with oxygen and that their amount in tissues changes markedly and instantaneously by the change in the functional state of vegetative nervous system and also by the administration of such substances as minerals and vitamins *etc.* It has also been found that the changes in their amount in tissues caused by various factors are intimately related to the functional state of those tissues. Hence their amount especially that of metalloporphyrin is a good indicator for the functional state of tissues.

In view of these findings a question arises whether the administration of such substances as being closely connected with life phenomena will cause the increase in the porphyrin bodies of tissues or it will cause the decrease. In order to answer this question a systematic investigation has been undertaken, using amino acids as the first step. Sulphur containing amino acids are known to increase the function of various tissues and especially their relation to the liver injury has recently been well-established (*2*). The mechanism of their action on the liver, however, is not yet completely elucidated. The present view is simply that the liver injury is caused by the swelling of liver cells due to the insufficient supply of cystine to the cells. But the mechanism of the development of swelling is unknown. It is considered that the behavior of glutathion (*2*) has in some way to do with the phenomenon. Moreover, as for such an organ as liver which metabolizes amino acids, it should be ascertained whether or not the excessive administration, especially the repeated administration in large amounts, of sulphur containing amino acids if overloads the liver to bring about its injury. It should also be found if such an administration is harmful to the tissues other than the liver. These problems are important from the standpoint of their clinical application. The present experiment is, therefore, confined to find out the influence of the administration of sulphur containing amino acids upon the distribution of porphyrin bodies in the body. The following experimental results will give explicit answers for the above-indicated problems.

EXPERIMENTAL

A portion of 0.2 ml. per 100 g. body weight of the aqueous solution of a sulphur containing amino acids was given subcutaneously once or several times (once daily) to healthy guinea pigs, showing normal increase in their body weight on the feed consisting of fresh grass and some vegetables, in early morning when the animals were hungry and at the end of a certain time thereafter the amount of porphyrin bodies contained in various tissues, blood, urine *etc.* were measured by Kôsaki's method (*1*, *3*, *4*). Either 3300 mg./dl.~33 mg./dl. hot water solutions of L-cystine hydrochloride or 3300 mg./dl.~33 mg./dl. aqueous solutions of L-cysteine hydrochloride or 3000 mg./dl.~30 mg./dl. aqueous solutions of DL-methionine were used. The concentration of methionine was so adjusted as to be eqimolar to cystine or cysteine.

The results of the experiment are shown in Tables I~VIII. The values obtained with untreated animals are mean values of normal guinea pigs already reported (*1*, *4*) and they are omitted in Table III *etc.* The experiments were performed in the year of 1951.

RESULTS AND DISCUSSION

As is clear from the tables, the metalloporphyrin content of all tissues except blood are markedly increased at the end of 30 minutes after the parenteral administration of the sulphur containing amino acids, although the increase varies with the kinds of amino acids and also with the amount administered. The porphyrin content of tissues *etc.* is not decreased, but in general rather increases. The administration of the sulphur containing amino acid may, therefore, be considered to increase the metalloporphyrin and the porphyrin of tissues, which can be conceived to be due mainly to the increased affinity of tissues for

TABLE I

Metalloporphyrin Content of Various Tissues after the Administration of Cysteine

To guinea pigs 5 mg. per 100 g. body weight of cystine is given subcutaneously. Porphyrin bodies content is expressed in γ per 100 ml. or per 100 g. wet weight.

Treatment	Animals			Date	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Urine
	No.	Body weight	Sex															
None	Average				**103**	**65**	**178**	**60**	**701**	**365**	**46**	**6**	**8**	**32**	**5**	**4**	**109**	**13**
At 30 min. after administration	1	380	♂	2/II				125	902	979	78	11	9	62	10	18	94	6
	2	390	♂	9/II	211	157		123		1409	136			147	21	38	100	
	3	375	♀	17/II	178	271	297	143	1095	1599	186		7	46	21	51		
	4	250	♀	27/II	158	105	314	203	1289	1407	274		50	150	31	127	107	
	5	315	♂	8/VI	182	225	658	98	962	1626	220		60	103		42	35	
	Average				**182**	**190**	**423**	**138**	**1062**	**1404**	**179**	**11**	**32**	**102**	**21**	**55**	**84**	**6**
At 3 hrs. after administration	6	355	♂	2/II	99	154	491	146	1457	1534	63	82	21	55	9	60	171	18
	7	320	♂	9/II		83	310			1089	55		31	77	10	49	80	
	8	380	♂	17/II	142	111	207	153	742	1446	174	46		159	22	25		
	9	275	♂	27/II	157	231	417	178	1114	1671	30		86	129	24	77	176	18
	Average				**133**	**145**	**356**	**159**	**1104**	**1435**	**80**	**64**	**46**	**105**	**16**	**53**	**142**	**18**
At 24 hrs. after administration	10	360	♂	3/II	86		269		401	1210		13	15					
	11	350	♂	10/II						1527	66		22	79	12	38	119	
	12	390	♀	20/II	114	130	234	80	266	595	150			46	28	78	108	38
	13	340	♂	28/II	79	154	135	144	604	1229	81	53	51	72	24	65	85	20
	Average				**93**	**142**	**213**	**112**	**424**	**1140**	**99**	**33**	**29**	**66**	**31**	**60**	**104**	**29**

TABLE II

Porphyrin Content of Various Tissues after the Administration of Cystine

Under the same condition as those in Table I.

Treatment	No. of Animals	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Bone marrow	Whole blood	Blood plasma	Bile	Urine
None	Av.	**2**	**2**	**4**	**1**	**10**	**21**	**2**	**2**	**2**	**2**	**2**	**7**	**15**	**15**	**51**	**2**	**9**	**8**
At 30 min. after administ-ration	1	2	2	4	1	6	11	1	1	2	3	2	6	8	17	32	3	6	2
	2	1	2	5	2	6	25	1	2	9	4	2	10	35	34	32	6		
	3	3	3	5	1	14	35	2		5	3	3	8	6	47	32	3	5	3
	4	2	3	8	1	23	26	3		2	5	4	14	33	14	18	2	8	3
	5	2	2	4	2	9	49	1	5	2	1	2	8	12	21	25	3	19	
	Av.	**2**	**2**	**5**	**1**	**12**	**29**	**2**	**3**	**4**	**3**	**3**	**9**	**19**	**27**	**28**	**3**	**10**	**3**
At 3 hrs. after administ-ration	6	2	3	3	1	13	33	1	3	3	2	2	6	9	30	19	2	4	15
	7	3	2	8		36	63		11	4	4	2	14	31	42	38	5	18	
	8	2	2	3	2	8	12	1	2	3	2	2	10	11	30	12	3	3	3
	9	2	2	3	2	18	56	2	9	2		3	14	50	41	17	2	14	6
	Av.	**2**	**2**	**4**	**2**	**19**	**41**	**1**	**6**	**3**	**3**	**2**	**11**	**25**	**36**	**22**	**3**	**10**	**8**
At 24 hrs. after administ-ration	10	1	2	1	1	7	18	3	4	2	1	2	3	10	15	51	2	5	6
	11	2	2	3	2	21	38	3	5	2	5	2	6	11	28	30	4	4	
	12	2	2	3	1	14	24	2		3	2	1	5	6		19	2	7	6
	13	3	4	6	3	14	33	4	11	3			3			32	2	6	2
	Av.	**2**	**2**	**3**	**2**	**14**	**26**	**3**	**7**	**2**	**3**	**2**	**4**	**9**	**22**	**33**	**2**	**6**	**5**

Table III

Metalloporphyrin Content of Various Tissues after the Administration of Cystine

To guinea pigs cystine is administered subcutaneously. In case of repeated administration 0.5 mg. per 100 g. body weight of cystine is given daily and the measurement is performed at 24 hrs. after the last administration. Porphyrin bodies content is expressed in γ per 100 ml. or per 100 g. wet weight.

Treatment	Animals				Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Urine
	No.	Body weight	Sex	Date														
After 30 min., 0.5 mg./100 g.	14	160	♂	27/IV	179	201	674	210		1194	145		47	38		41		
	15	300	♂	9/VI	173	120	378	144	485	1198	121				11	34	53	
	Average				**176**	**160**	**526**	**177**	**485**	**1196**	**133**		**47**	**38**	**11**	**38**	**53**	
After 3 hrs., 0.5 mg./100 g.	16	350	♀	18/V	**124**	**108**	**236**	**56**	**875**	**1351**	**203**		**6**	**24**	**7**	**22**	**147**	**4**
After 24 hrs., 0.5 mg./100 g.	17	360	♂	28/IV	**126**	**167**	**201**	**127**	**783**	**895**	**131**	**57**	**10**	**94**	**30**	**48**	**73**	**14**
After 30 min., 0.1 mg./100 g.	18	340	♂	15/VI	**179**	**248**	**442**	**186**	**1822**	**2813**	**218**		**71**	**136**	**18**	**41**	**52**	
After 30 min., 0.05mg./100g.	19	350	♂	23/V	85	64	135	63	383	1451	153	99	8	26	17	25	55	14
	20	320	♂	15/VI	122	137	135		1628	1425	155	30	11	66	13	48	73	14
	Average				**104**	**100**	**135**	**63**	**1006**	**1438**	**154**	**64**	**10**	**46**	**15**	**36**	**64**	**14**
2 times adm.	21	315	♂	27/VI	**60**	**90**	**106**	**71**	**585**	**465**	**16**	**189**	**41**	**47**	**40**	**111**	**168**	
3 times adm.	22	340	♂	18/VI	**83**	**116**	**62**	**64**	**963**	**1237**	**174**	**40**	**33**	**21**	**18**	**56**	**100**	
7 times adm.	23	320	♂	12/VI	132	74	276	146	511	1264	88	200	8	72	196	515	123	
	24	340	♂	6/VII	79	108	549	324	1533	960	17	15	13	349	5	49	60	
	25	500	♀	30/VIII	218	208	532	200	1172	759	203		15	6	11	62	49	13
	Average				**143**	**130**	**452**	**223**	**1072**	**994**	**103**	**108**	**12**	**142**	**71**	**209**	**77**	**13**

TABLE IV

Porphyrin Content of Various Tissues after the Administration of Cystine

Under the same condition as those in Table III.

Treatment	No. of Animals	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Bone marrow	Whole blood	Blood plasma	Bile	Urine
After 30 min., 0.5 mg./100 g.	14	2	2	6	8	24	26	2		1	2	2	11	20	39	22	3	10	3
	15	2	4	4	2	15	30	2	6	4	2	1	13	17	24	35	3		8
	Av.	2	3	5	5	20	28	2	6	2	2	2	12	18	32	28	3	10	6
After 3 hrs., 0.5 mg./100 g.	16	2	2	4	2	11	26	2		4	4	3	17	35	27	24	2		3
After 24 hrs., 0.5 mg./100 g.	17	2	2	1	1	12	27	2	5	2	6	2	5	26	26	30	2	5	3
After 30 min., 0.1 mg./100 g.	18	2	2	3	1	10	29	3	3	3	4	2	11	12	39	31	3	6	10
After 30 min., 0.05 mg./100 g.	19	1	1	4	1	7	14	1	2	1	1	2	11	9	13	12	1	3	3
	20	2	2	6	1	10	15	3	3	2	1	2	9	13	42	20	2	9	3
	Av.	2	2	5	1	8	14	2	2	2	1	2	10	11	28	16	2	6	3
2 times adm.	21	2	2	6	2	16	10	6	10	1	2	1	5	8	11	35	1	11	7
3 times adm.	22	1	2	4	1	12	22	2	2	2	3	1	5	10	27	22	3	10	11
7 times adm.	23	2	2	2	2	3	14	2	2	2	2	1	4	5	9	11	2		5
	24	3	2	4	3	8	29	1	2	1	1	5	4	16	20	34	2	24	
	25	2	2	4	2	9	5	1		4	4	2	10	7	34	32	3	10	4
	Av.	2	2	3	2	7	16	1	2	2	2	3	6	9	21	26	2	17	4

TABLE V

Metalloporphyrin Content of Various Tissues after the Administration of Cysteine

To guinea pigs cysteine is given subcutaneously. Porphyrin bodies content is expressed in γ per 100 ml. or per 100 g. wet weight.

Treatment	Animals			Date	Lives	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Urine
	No.	Body weight	Sex															
After 30 min., 5 mg./100 g.	26	470	♂	2/VIII	**221**	**158**	**427**	**100**	**1019**	**1223**	**111**	**30**	**32**	**42**	**28**	**91**	**130**	**38**
After 3 hrs., 5 mg./100 g.	27	330	♂	17/VII	**103**	**97**	**182**	**86**	**670**	**1903**	**49**	**31**	**2**	**26**		**8**	**233**	
After 24 hrs., 5 mg./100 g.	28	540	♂	22/VIII	**78**	**158**	**254**	**126**	**874**	**586**	**59**		**2**	**4**	**1**	**7**	**21**	**4**
After 30 min., 0.5 mg./100 g.	29	340	♂	20/VI	**243**	**242**	**614**	**296**	**1501**	**734**	**166**	**352**	**44**	**49**	**42**	**91**	**32**	
After 30 min., 0.1 mg./100 g.	30	245	♂	23/VI	**242**	**159**	**314**	**127**	**1229**	**1071**	**86**	**425**	**25**	**111**	**6**	**12**	**54**	**3**
After 30 min., 0.05mg./100g.	31	230	♀	22/VI	**183**	**264**	**597**	**224**	**1610**	**2563**	**140**		**87**	**144**	**197**	**295**	**186**	**45**

TABLE VI

Porphyrin Content of Various Tissues after the Administration of Cysteine

Under the same condition as those in Table V.

Treatment	No. of Animals	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Bone marrow	Whole blood	Blood plasma	Bile	Urine
After 30 min., 5 mg./100 g.	26	**1**	**1**	**4**	**1**	**7**	**10**	**3**	**1**	**3**	**2**	**3**	**15**	**19**	**32**	**7**	**1**	**12**	**13**
After 3 hrs., 5 mg./100 g.	27	**2**	**2**	**3**	**2**	**2**	**41**	**2**	**4**	**2**	**2**	**2**	**9**	**11**	**22**	**6**	**2**	**8**	**12**
After 24 hrs., 5 mg./100 g.	28	**1**	**2**	**2**	**1**	**8**	**11**	**2**		**4**	**3**	**3**	**12**	**7**	**8**	**13**		**7**	**7**
After 30 min., 0.5 mg./100 g.	29	**2**	**2**	**4**	**2**	**18**	**12**	**3**	**4**	**1**	**2**	**1**	**5**	**16**	**7**	**15**	**2**		**4**
After 30 min., 0.1 mg./100 g.	30	**1**	**1**	**3**	**1**	**19**	**22**	**1**	**3**	**2**	**1**	**1**	**5**	**6**	**8**	**21**	**1**	**3**	**2**
After 30 min., 0.05mg./100g.	31	**1**	**2**	**4**	**2**	**11**	**25**	**3**		**1**	**1**	**2**	**5**	**16**	**28**	**13**	**4**	**2**	**5**

TABLE VII

Metalloporphyrin Content of Various Tissues after the Administration of Methionine

To guinea pigs methionine is given subcutaneously. In case of repeated administration 6 mg. per 100 g. body weight is given daily and the measurement is performed at 24 hours after the last administration. Porphyrin bodies content is expressed in γ per 100 ml. or per 100 g. wet weight.

Treatment	Animals			Date	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Urine
	No.	Body weight	Sex															
After 30 min., 6 mg./100 g.	32	410	♂	6/III	75	143	251	116	624	953	64	32	32	22		17	46	69
	33	480	♂	9/III	138	140	139	112	593	441	42	10	10		12		39	43
	34	350	♂	14/III	97	115	182	131	385	655	73	73	73	12	23	37	81	176
	Average				**103**	**133**	**191**	**120**	**417**	**683**	**60**	**60**	**38**	**17**	**12**	**27**	**55**	**96**
After 3 hrs., 6 mg./100 g.	35	610	♂	6/III	162	105	133	186	417		195	85	41	33	33	68	56	15
	36	460	♂	9/III	123	98	181	123	746	616	127	33	44	41	24	132	87	32
	37	440	♀	14/III	139	160	162	135		743	141		48			133	62	9
	Average				**141**	**121**	**159**	**148**	**582**	**680**	**154**	**59**	**44**	**37**	**28**	**111**	**68**	**19**
After 24 hrs., 6 mg./100 g.	38	550	♂	21/IV	130	193	693	215	693	679	45	23	29	4		90	67	4
	39	220	♂	25/IV	105	114	395	60	991	945	51		27	13	7	55	139	6
	40	330	♂	10/V	131	129	163	117	334	833	88	60	32		2		35	5
	Average				**122**	**145**	**417**	**131**	**673**	**819**	**61**	**42**	**29**	**8**	**4**	**72**	**80**	**5**
After 30 min., 1.2 mg./100 g.	41	565	♂	20/VIII	**142**	**168**	**404**	**126**	**1081**	**1101**	**90**	**10**	**15**	**68**	**5**	**13**	**11**	**4**
After 30 min., 0.6 mg./100 g.	42	290	♂	26/V	**156**	**82**	**300**	**88**	**771**	**1729**	**126**	**56**	**15**	**58**	**11**	**33**	**102**	
After 30 min., 0.12 mg./100 g.	43	330	♂	7/VII	237	187	603	125	2973	2822	29	109	12	138	4	19	37	12
	44	260	♂	7/IX	128	169	202	112	2969	2299		514	85	163	61	19	237	
	Average				**182**	**178**	**402**	**118**	**2971**	**2560**	**29**	**312**	**48**	**150**	**32**	**19**	**137**	**12**
After 3 hr., 0.12 mg./100 g.	45	440	♀	7/IX	**201**	**235**	**369**	**181**	**1343**	**964**	**182**		**102**	**49**	**24**	**39**	**143**	**31**
After 24 hr., 0.12 mg./100 g.	46	490	♀	8/IX	**285**	**238**	**245**	**161**	**942**	**534**	**172**		**76**	**119**	**25**	**203**	**119**	**37**
After 30 min., 0.06 mg./100 g.	47	460	♂	13/VII	**145**	**182**	**229**	**166**	**679**	**952**	**31**	**4**	**4**	**13**	**3**	**27**	**22**	**7**
3 times adm.	48	485	♀	16/VIII	**138**	**63**	**269**	**69**	**325**	**459**	**69**		**3**	**37**	**14**	**22**	**47**	
7 times adm.	49	425	♀	31/VIII	**324**	**229**	**446**	**200**	**2035**	**601**	**73**		**2**	**4**	**1**	**9**	**10**	

TABLE VIII

Porphyrin Content of Various Tissues after the Administration of Methionine

Under the same condition as those in Table VII.

Treatment	No. of Animals	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Bone marrow	Whole blood	Blood plasma	Bile	Urine
After 30 min., 6 mg./100 g.	32	2	1	1	1	12	57	1	1	2	2	1	8	12	24		2	10	
	33	4	3	5	2	15	24	2	2	2	3	2	10	8	23	27	3	3	6
	34	2	1	3	2	5	12	1	1	2	2	1	17	18	29	30	4	13	5
	Av.	**3**	**2**	**3**	**2**	**9**	**31**	**1**	**1**	**2**	**2**	**1**	**12**	**13**	**25**	**28**	**3**	**8**	**6**
After 3 hrs., 6 mg./100 g.	35	2	2	2	2	7	9	1	1	2	3	3	11	8	24	20	3	3	5
	36	2	1	2	2	9	10	1	2	2	3	2	5	9	24	31	2		7
	37	2	2	5	2	4	9	1		1	1	2	4	7	16	22	3	3	3
	Av.	**2**	**2**	**3**	**2**	**7**	**9**	**1**	**2**	**2**	**2**	**2**	**7**	**8**	**21**	**24**	**3**	**3**	**5**
After 24 hrs., 6 mg./100 g.	38	1	2	3	1	8	10	1	1	2	1	1	4	3	26	16	2	4	1
	39	1	1	4	2	9	35	1	7	1	1	1	14	1	15	20	1	4	2
	40	1	3	3	2	7	21	5	6	2	2	2	10	12	18	17	2	**10**	4
	Av.	**1**	**2**	**3**	**2**	**8**	**22**	**2**	5	**2**	**1**	**1**	**9**	**5**	**20**	**18**	**2**	**9**	**2**
After 30 min., 1.2 mg./100 g.	41	**2**	**3**	**2**	**3**	**8**	**20**	**3**	**1**	**6**	**5**	**3**	**11**	**11**	**7**	**23**		**3**	**3**
After 30 min., 0.6 mg./100 g.	42	**2**	**1**	**3**	**2**	**9**	**31**	**3**	**5**	**2**	**2**	**1**	**6**	**15**	**35**	**35**	**9**	**100**	**11**
After 30 min., 0.12 mg./100 g.	43	1	1	1	1	7	9	1	1	1	1	1	3	8	13	37	2	7	1
	44	2	1	7	2	31	69	5	15	4	3	3	10	48	33	32	2	22	9
	Av.	**2**	**1**	**4**	**2**	**19**	**39**	**3**	**8**	**2**	**2**	**2**	**6**	**28**	**23**	**34**	**2**	**14**	**5**
After 3 hrs., 0.12 mg./100 g.	45	**2**	**2**	**4**	**3**	**17**	**14**	**3**		**2**	**4**	**4**	**24**	**23**	**27**	**44**	**4**	**17**	**3**
After 24 hrs., 0.12 mg./100 g.	46	**3**	**4**	**3**	**3**	**7**	**10**	**2**		**13**	**2**	**1**	**7**	**10**	**25**	**38**	**2**	**9**	**7**
After 30 min., 0.06 mg./100 g.	47	**1**	2	5	**1**	7	**12**	**1**	**1**	**1**	2	**1**	6	6	**11**	2		**13**	**1**
3 times adm.	48	**1**	**2**	**4**	**2**	**8**	**22**	**2**		**1**	**2**	**1**	**3**	**3**	**20**	**18**	**1**	**2**	**6**
7 times adm.	49	**1**	**1**	**3**	**1**	**10**	**7**	**2**		**2**	**3**	**2**	**7**	**10**	**21**	**28**	**2**	**3**	**6**

porphyrin bodies especially for metalloporphyrin. As the increase in the metalloporphyrin content of a tissue will cause the increased function of that tissue (*1*), it can be concluded that there is such an aspect in the physiological actions of sulphur containing amino acids to increase the cellular activities as to be explained by the increase in the metalloporphyrin of cells. As for the liver, the development of the swelling and subsequent injury or necrosis in the case of the deficient supply of the sulphur containing amino acids, which increase liver metalloporphyrin, can be well interpreted as being due to the disturbance in the intracellular oxidation of liver cells caused by the lack of metalloporphyrin.

Accordingly, the administration of sulphur containing amino acids may be considered to be an adequate measure for the treatment of such diseases as resulted from the decrease in the metalloporphyrin content of tissues, for example lead poisoning or sulfonal porphyria (*1*, *5*), of liver injury and also of such a case in which the functional increase of a certain organ or organs is expected.

The changes in the porphyrin bodies content of tissues after the administration of different sulphur containing amino acids are as follows: After the administration of cystine (5 mg./100 g. body weight) the metalloporphyrin in various tissues increases markedly, while the porphyrin bodies of urine and the porphyrin of whole blood decrease. Such changes become most marked at the end of 30 minutes after the administration in liver, kindneys, heart, and intestine, while in other tissues at the end of about 3 hours. At the end of 24 hours almost all of them disappear and some of them show tendencies to disappear. The increase of metalloporphyrin is marked especially in muscle, suprarenals and brain. The metalloporphyrin of liver and spleen decreases at the end of 24 hours after the administration. This point will be discussed later.

After the administration of cysteine (5 mg./100 g. body weight), the changes are almost similar to those of cystine, but the increase of metalloporphyrin is more marked in liver, spleen, suprarenals *etc.* than in the case of cystine administration, and in liver, kidneys, heart, intestine, skin, lungs and brain it reaches to its maximum at the end of 30 minutes after the administration. At the end of 24 hours the decrease of liver-metalloporphyrin is more marked with cysteine than with cystine. The metalloporphyrin content of skin and bone also decreases. The changes occur more markedly and in general more quickly with cysteine than with cystine. This indicates that cystine acts upon the porphyrin bodies after it has been transformed into cysteine.

After the administration of methionine (6 mg./100 g.), the changes that take place are similar to those cases when cystime or cysteine are used, nevertheless the increases in the metalloporphyrin content in those tissues are generally less marked with methionine than with cystine except in cerebellum. The time required to reach the maximum increase is generally 3 hours, but 24 hours in kidneys and heart. At the end of 24 hours after the administration its content decreases only in lungs but not in liver. Namely, the increase in metalloporphyrin occurs more slowly in general and less markedly, and its decrease is less marked with metnionine than with cystine. The fact that the action of methionine is less marked and developes more slowly than that of cystine or cysteine, suggests that methionine would exert its action on the porphyrin bodies of tissues after it has been changed to some extent.

The results obtained at the end of 30 minutes after the administration of various amounts indicate that all of the sulphur containing amino acids examined cause the increase of the metalloporphyrin content in all tissues and there exists an optimum amount of the amino acid administered in relation to the increase in the metalloporphyrin content. Although the optimum amount differs slightly with different tissues, it is 0.1 mg./100 g. body weight with cystine and methionine, and 0.05 mg./100 g. body weight with cysteine. In general, the increase in the metalloporphyrin after the administration of the optimum amount is most marked with cysteine, next with cystine, and least marked with methionine, although the latter two show difference only slightly. In intestine the increase is most marked with cystine and in spleen most marked with methionine.

Although the metalloporphyrin of liver decreases at the end of 24 hours after the administration of 5 mg./100 g. of cystine, it does not become less than normal at the end of 24 hours after the administration of 0.5 mg./100 g. of cystine and that of other tissues is increased in general. The time needed for the maximum increase of the metalloporphyrin is longer in intestine and shorter in skin when 0.5 mg. is administered than when 5 mg. is administered, but the type of the changes is almost equal in both cases. By the administration of 0.12 mg./100 g. of methionine the increase of metalloporphyrin is generally more marked and reaches to its maximum more slowly than by the administration of 6 mg./100 g., with exception of heart and suprarenals, the metalloporphyrin contents of which reach their maxima sooner (30 minutes) and are smaller (at the end of 24 hours after the administration).

The above-indicated results show that there is an optimum amount of the amino acids administered for the increase of the metalloporphyrin content in tissues, that is, the increase of metalloporphyrin becomes less marked in case when an amount larger than this optimum is administered. For the clinical application of sulphur containing amino acids this fact suggests that they should not be over-loaded.

Moreover, cystine when given in a larger amount causes at the end of 24 hours after the administration a decrease of the liver metalloporphyrin, namely the decrease of the liver functions, which is an undesirable effect. At the single dosage of 0.5 mg./100 g., cystine has been found to bring about no decrease of the liver metalloporphyrin, but a marked decrease has been observed in 3 of 5 cases when repeatedly administered. It may, therefore, be concluded that cystine given repeatedly even in a small dose as 0.5 mg./100 g. causes such an unfavourable effect on the liver as to decrease its metalloporphyrin. This result will explain the well-known fact that cystine, when given in excess doses (*5*), and often even in not too great excess of the physiologic norm (*6*), fails to prevent but on the contrary promotes the development of acute diffuse hepatic necrosis and of liver cirrhosis (*2*).

The metalloporphyrin of the tissues other than liver do not decrease in general after the repeated administration of cystine, but their increases are less marked than those after a single administration.

Clinically the repeated administration of cystine, therefore, should be avoided, while a single administration of a certain small amount of cystine will bring about a good effect.

As for methionine, even when it is repeatedly administered in such a large dose as 6 mg./100 g. a gradual increase of metalloporphyrin in liver and no decrease in other tissues except skin, cerebellum and bone has been observed. Thus, the methionine may be repeatedly administered in such a dose as 0.12 mg./100 g. without any unfavourable effect and in fact this is the most effective way to increase the metalloporphyrin of liver. This will explain the therapeutic effect of methionine on the liver injury and especially on liver cirrhosis, which has recently been much discussed.

In conclusion, the physiological action of sulphur containing amino acids can be explained by the change of the amount of the porphyrin bodies, especially metalloporphyrin in tissues, which is caused by the administration of the sulphur containing amino acids. Cystine or cysteine acts injuriously even in not too great excess but methionine exerts its most favourable effect when given in certain small doses.

SUMMARY

1. Sulphur containing amino acids in general increase the metalloporphyrin of tissues markedly, there being no decrease in the porphyrin of tissues.

2. The increase of tissue metalloporphyrin becomes most marked at the end of a certain time after the administration and then it gradually decreases.

3. The change in tissue metalloporphyrin takes place most markedly and rapidly with cysteine and least markedly and slowly with methionine.

4. With cysteine the increase in tissue metalloporphyrin is most marked when given in such does as 0.05 mg./100 g. and with cystine and methionine 0.1 mg./100 g. and the change is less marked when they are given in larger doses.

5. At the end of 24 hours after the administration of cystine or cysteine in such a dose as 5 mg./100 g. liver metalloporphyrin decreases beyond the normal value and the decrease is more marked with cysteine.

6. Repeated administration of cystine in dose of 0.5 mg./100 g. causes often the decrease of liver metalloporphyrin.

7. The repeated administration of methionine even in such a large dose as 6 mg./100 g. causes no decrease of liver metalloporphyrin.

8. The above results indicate that the physiological action of sulphur containing amino acids manifests itself through the change in the metalloporphyrin of tissues.

REFERENCES

(*1*) Kôsaki, T., *J. Mie Med. Coll.*, **1**, 85 (1950) ; **2**, 85 (1951)

(*2*) Himsworth, H.P., *The liver and its diseases*, Blackwell Scientific Publicatiens, Oxford, 2 nd ed., (1950)

(*3*) Kôsaki, T., *Saishin-Igaku*, **2**, 513 (1947) (in Japanese)

(*4*) Kôsaki, T., and Kawai, S., *Saishin-Igaku*, **4**, 97 (1949) (in Japanese)

(*5*) Earle, D.P., Jr., and Victor, J., *J. Exp. Med.*, **39**, 828 (1927)

(*6*) Curtis, A.C., and Newburgh, L.H., *Arch. int. Med.*, **39**, 829 (1927)

The Journal of Biochemistry, Vol. 39, No. 4.

ON THE INFLUENCE OF THE ADMINISTRATION OF AMINO ACIDS UPON THE DISTRIBUTION OF PORPHYRIN BODIES IN TISSUES. II. THE INFLUENCE OF THE ADMINISTRATION OF ε-AMINO CAPROIC ACID AND RELATED SUBSTANCES

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(Received for publication, May 8, 1952)

In the first paper (*1*) dealing with the physiological action of various amino acids indicated by the measurement of porphyrin bodies (general term for porphyrin and metalloporphyrin) in various tissues it has been reported that sulphur containing amino acids are dominant factors for the increase of tissue-porphyrin bodies and also that the physiological action of sulphur containing amino acids manifests itself through the change of the metalloporphyrin of tissues.

ε-Amino caproic acid* has been found by the researchers of the Toyo Rayon Company to stimulate the growth of higher plants and yeast, and also by the members of Prof. H. Yamada's Pharmacological Department of the Medical Faculty of Kyoto University to stimulate the diuresis (*2*), the growth of cultured fibroblasts (*3*) far markedly than norleucine (*2*, *3*), and the mitosis of Yoshida sarcoma cells to some extent (*4*). As for the physiological action of related substances there are only few reports, namely that lysine has antibacterial action (*5*) besides diuretic action (*6*) and stimulates the non-enzymatic decarboxylation of oxalic acid (*7*), and that norleucine suppresses the growth of bacteria (*8*), stimulate the oxygen uptake of minced normal liver (*9*) and the non-enzymatic decarboxylation of oxalic acid (*7*), and exerts almost no harmful effect on rats except transient increase of body-temperature when given in large doses (*8*).

Thereupon the authors have planned to investigate the changes of tissue porphyrin bodies by the administration of ε-amino caproic acid and related substances, caproic acid, norleucine, and lysine, with the purpose of revealing the physiological action of ε-amino caproic acid in comparison with those of related substances and also of finding some clue to the elucidation of the relationship between the physiological action and the chemical structure. Interesting results have thus been obtained, the outline of which will be described in the present report.

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* The preparation is the by-product of Rayon industry and has been made by the Toyo Rayon Company.

EXPERIMENTAL

A portion of 0.2 mg./100 g. body weight of caproic acid (0.9 mg./ml. aqueous solution), DL-norleucine (1.0 mg./ml. aqueous solution), ε-amino caproic acid (1.0 mg./ml. aqueous solution) or DL-lysine monohydrochloride (1.4 mg./ml. aqueous solution) was administered subcutaneously to guinea pigs and at the end of 30 minutes after the administration the amount of porphyrin bodies contained in tissues *etc.* was determined by means of Kôsaki's method (*10*, *11*, *12*). The results are shown in Tables I and II. The values of control animals are the mean values of normal guinea pigs already reported (*11*, *12*).

RESULTS AND DISCUSSION

ε-Amino caproic acid causes marked increases in the metalloporphyrin of various tissues other than intestine, skin, lungs, cerebrum and bone, but marked decreases in the porphyrin of whole blood and the porphyrin bodies of urine. Judging from this fact, ε-amino caproic acid is a factor that increases tissue metalloporphyrin, which is conceived to be due mainly to the increased affinity of tissue for metalloporphyrin and also a factor that reduces porphyrinuria. Owing to such a property of ε-amino caproic acid it is considered to participate in stimulating the growth of plants and the multiplication af certain cells. In view of this fact its medical application for the treatment of diseases, the essential feature of which is the decrease of tissue metalloporphyrin, is possible. It is worthy of note that the action of ε-amino caproic acid causing the increase of the tissue metalloporphyrin is especially marked and is equal or rather superior to that of methionine or cystine (*1*) in such organs as liver, kidneys and skeletal muscle, where the decrease of metalloporphyrin is the subject of interest at most.

Caproic acid acts in general on tissue metalloporphyrin rather as a decreasing factor although it sometimes acts increasingly. The changes in the metalloporphyrin of tissues caused by caproic acid are quite similar to those caused by mineral acid (*12*) except in the cases of the

TABLE I

Metalloporphyrin Content of Various Tissues after the Administration of ε-Amino Caproic Acid and Related Substances

Guinea pigs. At 30 min. after the subcutaneous injection of caproic acid (0.18 mg. per 100 g. body weight), norleucine (0.2 mg. per 100 g. body weight), ε-amino caproic acid (0.2 mg. per 100 g. body weight) or lysine (0.22 mg. per 100 g. body weight). Porphyrin bodies content is expressed in γ per 100 ml. or per 100 g. wet weight.

Treatment	Animals			Date	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Urine
	No.	Body weight	Sex															
None	Average				**103**	**65**	**178**	**60**	**701**	**365**	**46**	**6**	**8**	**32**	**5**	**4**	**109**	**13**
Caproic acid given	1	510	♂	14/XII, 1951	49	95	107	146	1110	688	86		11	11	4	26	44	
	2	390	♀	15/XII, 1951	55	127		79		468	57			24	2	27	51	5
	3	510	♂	18/ I , 1952	103	49	75	145	1437	398	41		3	6	3	26	23	6
	4	320	♀	5/ II , 1952	67		127	111	647	317			3	6	4	15	59	
	Average				**69**	**90**	**103**	**120**	**1065**	**486**	**61**		**6**	**12**	**3**	**24**	**44**	**6**
Nor-leucine given	5	340	♂	14/XII, 1951	90	146	101	63	3689	1114	29	17	12	56	1	15		13
	6	570	♂	21/XII, 1951			108	62		1403	33	11	5		3	29	30	5
	7	450	♂	11/ I , 1952	64	75	225	94	1885	485	12		3		2	11	83	5
	8	350	♂	5/ II , 1952	90	132	114	141	1569	456	29		2	35	3	21	29	7
	Average				**81**	**118**	**137**	**90**	**2381**	**865**	**26**	**14**	**6**	**46**	**2**	**19**	**47**	**8**
ε-Amino caproic acid given	9	350	♂	28/XI, 1951	425	327	98	75		632	72	6	5	7		8		3
	10	750	♂	7/XII, 1951	281	80	423	364	677		35	3	11	10	4	21		3
	11	500	♂	11/ I , 1952		79	157	233	1328	2138		10	5		3	16	40	4
	12	550	♂	18/ I , 1952	226	446		58	996	886	20	12	8	7	7	19	22	4
	13	430	♂	9/ II , 1952	102	115	147	64	1035	1056	40	9	4	5	3	4	31	
	Average				**259**	**209**	**206**	**159**	**1009**	**1178**	**42**	**8**	**7**	**7**	**4**	**16**	**31**	**4**
Lysine given	14	260	♂	12/XII, 1951	112	333	148			553	13	9	5	17	6	30	55	3
	15	540	♀	19/XII, 1951	124	464	79	124	2307					10	9	33	26	2
	16	610	♀	12/ I , 1952	179	121	84	81	1889	458	14		7	7	2	11	36	5
	17	350	♂	4/ II , 1952	189		153	62	1194	790	4	1	5	13	4	13	152	
	Average				**151**	**306**	**116**	**89**	**1797**	**600**	**10**	**5**	**6**	**12**	**5**	**22**	**67**	**3**

TABLE II

Porphyrin Content of Various Tissues after the Administration of ε-Amino Caproic Acid and Related Substances

Under the same condition as those in Table I.

Treatment	No. of Animals	Liver	Kidneys	Heart	Skeletal muscle	Spleen	Suprarenals	Intestine	Testicles	Skin	Lungs	Cerebrum	Cerebellum	Bone	Bone marrow	Whole blood	Blood plasma	Bile	Urine
None	Av.	**2**	**2**	**4**	**1**	**10**	**21**	**2**	**2**	**2**	**2**	**2**	**7**	**15**	**15**	**51**	**2**	**9**	**8**
Caproic acid given	1	1	1	1	1	5	6	1	1	4	2	1	4	7	27		3	5	8
	2	5	1	10	3	17	26	3		2	2	1	5	15	28	75	3	20	6
	3	3	2	2	2	12	15	1	2	2	2	2	4	12	9	25	2	7	2
	4	3	3	8	3	26	21	4		3	4	3	14	19	27	35	4	21	7
	Av.	**3**	**2**	**5**	**2**	**15**	**17**	**2**	**2**	**3**	**3**	**2**	**7**	**13**	**23**	**45**	**3**	**13**	**6**
Norleucine given	5	3	5	3	3	9	14	2	2	2	4	4	13	27	19	29	1	9	4
	6	4	1	2	3	7	11	1	1	2	6	2	8	8	20	23	3	13	4
	7	2	3	3	1	8	12	1		3	4	2	7	13	15	20	3		2
	8	1	2	3	1	21	28	1	1	3	2	2	5	17	49	12	3	10	4
	Av.	**3**	**3**	**3**	**2**	**11**	**16**	**1**	**1**	**3**	**4**	**3**	**8**	**16**	**26**	**21**	**3**	**11**	**4**
ε-Amino caproic acid given	9	1	2	2	1	17	35	3	1	1	5	1	4	42	16	13	3	15	5
	10	3	4	2	4	8	11	4	1	2	1	1	5	6	12	30	3	5	3
	11	3	1	1	1	3	13	3	1	2	3	2	15	12		12	2	4	2
	12	3	1	1	2	8	14	1	1	2	3	1	8	10	20	21	3	13	3
	13	2	1	4	3	18	27	3	1	2	4	2	14	10	25	23	4	4	4
	Av.	**2**	**2**	**2**	**2**	**11**	**20**	**3**	**1**	**2**	**3**	**1**	**9**	**16**	**18**	**20**	**3**	**8**	**3**
Lysine given	14	4	3	9	4	13	33	5	9	2	2	1	6	6	35	14	1		5
	15	4	3	4	5	26	7	3		1	1	1	6	4	5	6	2	6	4
	16	4	2	3	4	9	7	3		3	3	3	8	10	18	40	3	9	11
	17	3	3	5	4	24	22	4	2	2	2	1	4	5		11	1	5	6
	Av.	**4**	**3**	**5**	**4**	**18**	**17**	**4**	**6**	**2**	**2**	**2**	**6**	**6**	**19**	**18**	**2**	**7**	**7**

decrease in the metalloporphyrin of heart and skin, and of the increase in the metalloporphyrin of intestine and suprarenals, nevertheless the direct comparison of both cases is difficult because of the difference in the method of administration.

The compound obtained by substitution of an amino group either to α-or ε-position of caproic acid differed markedly from caproic acid in the effect upon tissue porphyrin bodies, especially metalloporphyrin, the difference in the effect varying in different tissues. The effect of α-substitution is as follows: marked increase in metalloporphyrin in spleen and lungs, slightly increase in kidneys and suprarenals, but no effect in liver, heart, skin and bone, and rather decrease in skeletal muscle, cerebrum and cerebellum, and a marked decrease in intestine. In other words, norleucine is rather a factor that decreases tissue metalloporphyrin in general. It can be concluded that norleucine in its free state exerts generally no favourable effect on various tissues and it is in agreement with the contemporary view.

With regard to tissue metalloporphyrin, the ε-substitution product markeply increases that in liver, kidneys and suprarenals, increases to some extent that in heart, skeletal muscle, skin, and cerebrum, is without effect for spleen and decreases that in intestine, lungs, cerebellum and bone.

Though the α- and ε-substitution products cause similar effects, their degrees differ markedly from each other as can be seen from the data for kidney for example. This is in good agreement with the results obtained by Yamada *et al.* (*2*). I may, therefore, be said that at least the diuretic effect of ε-amino caproic acid and related substances is mainly due to the increase in the metalloporphyrin of kidney.

It is interesting to note that there is a marked difference in the effect on tissue metalloporphyrin according to the position of the amino group substitution. Especially when an amino group is substituted to the non-polar end of caproic acid the effect of caproic acid in decreasing tissue metalloporphyrin is depressed.

In case two amino groups are substituted to both α- and ε-positions of caproic acid the effect on tissue metalloporphyrin is generally the algebraic sum of those of α- and ε-substitutions. This can be obviously seen in kidneys and intestine. In some tissues it is dependent mainly on the effect of either one, the effect of α-substitution being predominant for the majority of tissues. In liver and suprarenals the increase of metalloporphyrin is much smaller than the sum. Lysine is, thus, generally inferior to ε-amino caproic acid as a metalloporphyrin increasing factor.

The above results indicate the existence of such an aspect of the physiological action of ε-amino caproic acid and its related substances as to be explained by the change in the function of tissues due to the change in the amount of the tissue porphyrin bodies which has been caused by those substances. The results also suggest yet unknown physiological actions of those substances. Further they offer some clue to the elucidation of the relationship between the physiological action and the chemical structure.

SUMMARY

1. ε-Amino caproic acid increases markedly the metalloporphyrin of various tissues other than intestine, skin, lungs, cerebrum and bone and decreases the porphyrin of whole blood and the porphyrin bodies of urine.

2. Caproic acid is, in general, a factor that decreases more or less tissue metalloporphyrin.

3. In case an amino group is substituted to α- or ε-position of caproic acid, marked difference is observed in the effect on tissue metalloporphyrin according to the position of the substitution. The substitution to ε-position causes an increase of the metalloporphyrin and the substitution to α-position bring about an increase in the cases of a half number of tissues investigated and a decrease in the cases of other half.

4. The effect of α, ε-diamino caproic acid on tissue metalloporphyrin is either the algebraic sum of the effects of α-substitution and of ε-substitution or depends mainly upon the effect of either one, especially on that of α-substitution.

5. There is such an aspect in the physiological action of ε-amino caproic acid and its related substances as to be explained by the change in the tissue metalloporphyrin.

REFERENCES

(*1*) Kôsaki, T., and Ikeda, T., *J. Biochem.*, **39**, 367 (1952)
(*2*) Watanabe, N., unpublished
(*3*) Shirasaki, K., *Medicine and Biology*, **16**, 363 (1950) (in Japanese)
(*4*) Shirasaki, K., unpublished.
(*5*) Jeney, E., *Hung. Acta Physiol.*, **1**, 142 (1948), cit. from *Chem. Abstr.*, **43**, 9145 (1949)
(*6*) Lotspeich, W.D., and Pitts, R.F., *J. Biol. Chem.*, **168**, 611 (1947)
(*7*) Bessman, S.P., and Layne, E.C., Jr., *Arch. Biochem.*, **26**, 25 (1950)
(*8*) Vacirca, F., *Attualita Chemioterap.*, (1944) 9, cit. from *Chem. Abstr.*,

41, 1327 (1947)

(*9*) Waterman, N., Berkhout, H.W., and Bos, C.J., *Enzymol.*, **12**, 206 (1947)

(*10*) Kôsaki, T., *Saishin-Igaku*, **2**, 513 (1947) (in Japanese)

(*11*) Kôsaki, T., and Kawai, S., *Saishin-Igaku*, **4**, 97 (1949) (in Japanese)

(*12*) Kôsaki, T., *J. Mie Med. Coll.*, **1**, 85 (1950); **2**, 85 (1951)

The Journal of Biochemistry, Vol. 39, No. 4.

ON THE RELATION BETWEEN THE RETINENE REDUCTASE AND FLAVIN CONTAINING ENZYME

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(Received for publication, May 31, 1952)

It was reported in previous papers (*1*) that the interconversion of the retinene$_1$ and vitamin A_1 in vitro can be performed by an enzyme system as already depicted by Wald *et al.* It consists of an apoenzyme, retinene reductase and DPN. Since the retinene reductase is a pyridine nucleotide enzyme it seems probable that a flavin containing enzyme acts as link between the retinene reductase and cytochrome system. In this connection it is interesting to note that Yagi (*2*, *3*) has confirmed recently the existence of flavin monouncleotide (FMN) in frog retina. If such a role of flavin enzyme is really the case it might be assumed that pteroylgutamic acid can decrease the action of retinene reductase, because it is claimed by Keith *et al* (*4*) and other investigators that flavinenzyme is specifically inhibited by pteroylglutamic acid. This paper presents some evidences for the participation of flavin enzyme to retinene$_1$-vitamine A_1 interconversion.

EXPERIMENTAL

Inhibition of Retinene Formation by Folic Acid—Four tenths ml. of homogenate which was freshly prepared frog retina were diluted with 0.6 ml. of phosphate buffer (pH 6.8) and mixed with various amounts of folic acid as shown in Table I. The mixture was incubated for 30 minutes under illumination at the room temperature, the reaction being interrupted by addition of methanol up to 50%. Then the whole mixture was extracted with pettoleum ether (boiling point 40—60°) and the absorption spectrum of the extract was measured in the Beckman D. U. spectrophotometer (Fig. 1).

The absorption maximum was found at 325 mμ, which shifted to 330 mμ in the mixture IV. The extinction at 325 mμ was plotted against the amount of added folic acid as shown in Fig. 2. It is clear

TABLE I

No. of mixture	Dark adapted retinal homogenate	Phosphate buffer at pH 6.8	Folic acid
	ml.	*ml.*	*mg.*
I.	0.4	0.6	
II.	0.4	0.6	1.5
III.	0.4	0.6	4.5
IV.	0.4	0.6	7.5

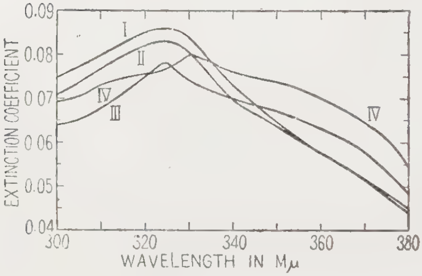

FIG. 1

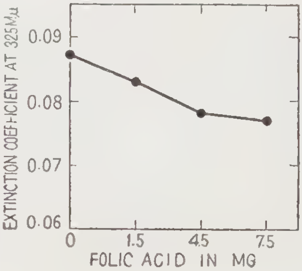

FIG. 2

in this experiment that formation of retinene is reduced by addition of folic acid.

Enhancement of Retinene Reductace by DPN and FAD—Into each of three test tubes were introduced 0.4 ml. of retinal homogenate, 0.6 ml of phosphate buffer (pH 6.8) and 1.0 ml. of 0.1 *N* hydroxylamine solution. The first tube served as control. To the second tube 4 mg. of DPN and to the third tube 4 mg. of DPN and 0.2 ml. of FAD solution (2–3 γ) were added. All tubes were incubated in darkness at the room temperature for two hours. Each reaction mixture was extracted with petroleum ether. The extract of the first tube showed a maximum at 330 mμ, the second at 335 mμ and the third at 340 mμ (Fig. 3). This fact indicates that in the presence of DPN and FAD the activity of the retinence reductase is fairly enhanced. On the contray ot FAD, addition of FMN did not cause

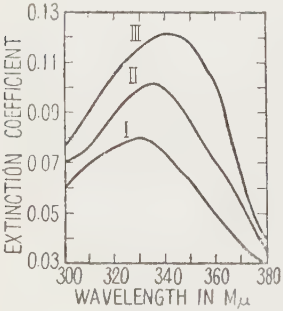

FIG. 3

any change in absorption maximum of the extract either in presence or absence of added DPN.

Inhibition of Retinene and Vitamin A Formation by Phlorhizin:—Five tenths ml. of 2.5 per cent phlorhizin solution were injected into hind leg muscles of frogs. The frogs were exposed to light for one to two hours and then kept overnight in darkness. The yield of retinene_1 and of vitamin A_1 of the frogs so treoted were compared with those of untreated frogs.

TABLE II

No. of mixture	Retinal homogenate	Phosphate buffer at pH 6.8	0.1 *N* NH_2OH
	ml.	*ml.*	*ml.*
I.	0.4	1.6	
II.	0.4	0.6	1.0
III.	0.4	1.6	
IV.	0.4	0.6	1.0

The reaction mixtures were prepored as shown in Table II, in which I and II represent the untreated retina and III and IV the phlorhizinized retina. The mixture I and III were immediately mixed with methanol to stop the reaction, while II and IV were kept in darkness for two hours at room temperature before the addition of methanol. The petroleum ether extracts from these four mixtures showed the absorption spectra as seen in Fig. 4. The result demonstrates that the yields of both retinene_1 and vitamin A_1 are decreased by phlorhizin treatment.

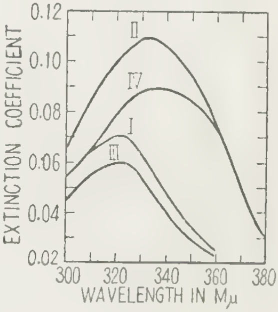

FIG. 4

DISCUSSION

Generally speaking, a flavin enzyme, diaphorase acts as a mediator between pyridine nucleotide dehydrogenases and cytochrome-cytochrome oxidase system. Since the retinene reductase has been shown to be a DPN containing enzyme, the existence or diaphorase in retina is indicated. The fact that pteroylglutamic acid inhibits the conversion of vitamin A_1 to retine_1 and that the addition of DPN alone or DPN and FAD enhance the reaction makes the theory more probable.

Acco̧ding to Yagi flavin in retina is exclusively of the form of FMN. This appears to be quite contradictory to the experimental results obtained here. But these is a posibility that FAD may synthesized from FMN *in vivo* with the cooperation of ATP in retina. The existence of ATP in retina, which Venkstern has reported in 1948 (*5*), suggests the occurrence of the following reaction:

$$\text{FMN}+\text{ATP} \leftrightharpoons \text{FAD}+\text{pyrophosphoric acid}$$

In 1935–38, Kalckar (*6*), DeConsilliis (*7*), and Oguchi (*8*) reported that the regeneration of rhodopsin is inhibited by phlorhizin, a poison of glycolysis. It is well known that retina is a unique tissue in performing intensive aerobic glycolysis though it is a mature and normal tissue. The experimental result that phlorhizin suppresses the regeneration of retinene suggests the intimate relationship between the aerobic glycolysis and the retinene formation, the energy to synthesize retinene from vitamin A being attained from the glycolysis. But the ultimate conclusion should be postponed until further experimental evidences will be obtained.

SUMMARY

1. Pteroylglutamic acid inhibited the regeneration of retinene$_1$ from vitamin A_1.

2. The yield of retinene$_1$ and vitamin A_1 was increased by adding DPN or DPN and FAD to the enzyme system.

3. The yield of retinene$_1$ and vitamine A_1 was decreased in phlorhizinized frogs.

REFERENCES

(*1*) Kojo, T., *Rinshoganka* (*Clinical ophthalmology, Japan*), **6**, 115 (1952); **6**, 244 (1952): **6**, 481, 485 (1952)
(*2*) Yagi, K., *J. Jap. Biochem. Soc.* (*Seikagaku*) **23**, 72 (1951)
(*3*) Yagi, K., *Jap. J. Physiol.*, **2**, 27 (1951)
(*4*) Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, **176**, 1095 (1948)
(*5*) Venkstern, T. V., *Biokhimina*, **14**, 238 (1948)
(*6*) Kalckar, *Nature* **136**, 872 (1935)
(*7*) DeConcilliis, cit. c.f.g.o., **34**, 150 (1935)
(*8*) Oguchi, T., *Acta Soc. Opthalm. Jap.*, **42**, 743 (1938)

The Journal of Biochemistry, Vol. 39, No. 4.

THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES. III. GLOBOSIDE, THE SUGAR-CONTAINING LIPID OF HUMAN BLOOD STROMA

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(Received for publication, June 13, 1952)

As shown in the first report of this series (*1*), the authors isolated palmityl sphingomyelin and an apparently new kind of glycolipid, designated hematoside, from the ether-insoluble lipid fraction of equine blood stroma. Hematoside gave purple coloration with Bial's orcinol reagent and was considered to be composed of lignoceric acid, sphingosine, D-galactose and prehemataminic acid in the proportion of 1 : 1 : 2 : 1.

Very soon after our report was published, Prof. Klenk reported that the obtained a surgar-containing lipid from the powdered human blood (*2*).

Since lignoceryl ganglioside had been found in the bovine spleen (*3*) and a close relationship between spleen and blood cells is generally accepted, he anticipated that a substance analogous to ganglioside, if not the same, would possibly be obtained.

The glycolipid obtained from human blood gave, however, no purple coloration when the Bial's test was applied; in other words, the presence of neuraminic acid or hemataminic acid (*4*) in this lipid was excluded. On hydrolysis, the substance proved to contain 29 per cent of fatty acid (mainly lignoceric acid), 20 per cent of sphingosine besides 40–41 per cent of sugar mixture. In the sugar mixture, chondrosamine was demonstrated along with galactose and glucose.

Very little was reported about sphingomyeline, which was obtained at the same time.

Thus, there is an essential discrepancy between the results of the German author with human blood and those of ours with equine blood stroma. At first sight, this discrepancy was considered to be most unusual. It seemed almost inconceivable that the difference of species would bring such a distinct difference in the composition of lipid.

We supposed that, since the source material used by Klenk was aged, as he mentioned in his paper, deterioration might have occured during a long period of storage; *i.e.*, hemataminic acid might have been converted to chondrosamine by enzymatic action.

Therefore, we obtained a large amount of fresh human blood corpuscles,* prepared stroma sample and extracted it with organic solvents by the same procedure as described in the first report.

During the preparation, human blood stroma proved to be much more soluble in water than the equine blood stroma, and as a result, a considerable amount of the sample might have been lost during the repeated washing procedure with water.

Two hundred and fifty grams of lyophilyzed stroma was extracted four times with methanol-ether at room temperature, and then was continuously extracted in a Soxhlet apparatus with chloroform-methanol, but the latter extract** was only in a small amount, easily turned resinous and was difficult to purify. However, the main portion of glycolipid proved to be already isolated in the methanol-ether extract.

From this crude glycolipid, 0.7 g. of colorless powder containing a trace of phosphorus was obtained. Contrary to our expectation, the substance gave negative Bial's test.

It differs, in this respect, from hematoside isolated from equine blood stroma ond seemed to be the same substance as Klenk's human blood glycolipid. However, because of the discrepancy of the analytical data obtained with our substance from those of Klenk's, we called this lipid "globoside" for convenience' sake. Since the substance formed perfectly round globules (spherocrystals) under microscope, the term "globoside" would be adequate (Fig. 1).

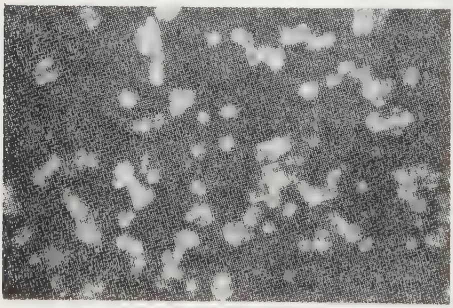

FIG. 1. Photomicrograph of globoside.

The analytical data obtained with the substance along with those of Klenk's glycolipid and hematoside are summarized in Table I.

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* 80 l. of fresh human blood corpuscles, residue from the preparation of lyophilyzed plasma for transfusion, was generously supplied by Dr. S. Kuniyuki of Nihon Seiyaku Co.

** In the case of equine blood stroma, hematoside is present in this faction.

TABLE I

	Globoside	Glycolipid (Klenk)	Hematoside
Carbon, %	64. 3	59. 82	61. 7
Hydrogen, %	10. 2	9. 95–10. 01	9. 6–9. 8
Nitrogen, %	2. 02–2. 07	1. 80–1. 86	2. 27
Carbohydrate, % (as galactose)	42–43	40. 5–40. 7	38–40
Hexosamine, % (as glucosamine HCl)	9. 03–9. 22	9. 7–10. 3	<0. 1
Hemataminic acid, % (or neuraminic acid)	0	0	21–22
Acetyl, %	2. 16–2. 82	—	1. 58
Specific Rotation	+10. 3°	—	−11. 9°
Melting Point	224°	234°	225–7°

As shown in the foregoing table, globoside contains practically the same amount of hexosamine as Klenk's substance, but the carbon content is considerably higher in the former. The specific rotation of globoside is in an opposite direction to that of hematoside. Volatile acid was detected after hydrolysis with sulfuric acid in both globoside and hematoside.

On hydrolysis, globoside liberated lignoceric and nervonic acids, the result being in accordance with that of Klenk. By the application of paper chromatography, galactose and glucose were detected together with chondrosamine. Since the spot of glucose on paper manifested less intense color than that of galactose, the ratio of these two sugars was determined by spectrophotometric analysis of the color developed with carbazole reagent. Accordingly, the amount of glucose proved to be extremely small as compared with that of galactose. The same was true about hematoside.

In view of the amounts of methyl ester of fatty acid (32.4 per cent), sphingosine-like base (25 per cent) and reducing value (42 per cent as galactose), hexosamine (10 per cent) and nitrogen (2.0 per cent) contents would correspond to 0.5 and 1.5 moles of globoside, respectively.

Comparing all these facts, we assume that globoside is a complex or a mixture of lignoceryl- (or nervonyl)-sphingosine-digalactoside* ($C_{54}H_{103}NO_{13}$) and lignoceryl- (or nervonyl)-sphingosine-acetylchondrosamine-digalactoside ($C_{62}H_{116}N_2O_{18}$). The values calculated agree closely with those estimated with globoside.

Calcd. for $C_{54}H_{103}NO_{13} \cdot C_{62}H_{116}N_2O_{18}$ (2151.94):
C 64.74, H 10.26, N 1.95, Sugar 41.9, Hexosamine-HCl 10.01, Acetyl 2.0.

Though the constituents of globoside are not yet completely confirmed in detail, because of the lack of material, its probable structure is thus assigned.

EXPERIMENTAL

Preparation of Material—80 l. of fresh human blood corpuscles, which was separated from serum by centrifugation, was washed with physiological saline, and 3 l. of the washed, packed red cells was added to 15 l. of tap water containing 50 ml. of glacial acetic acid. The supernatant hemoglobin solution was siphoned off from the pink-colored precipitate. Washings with tap water were repeated, but contrary to the case of equine blood stroma, appreciable amount of stroma dissolved away during washing procedures. Therefore, the samples were washed in the centrifuge-tube, water was removed in a low-temperature evaporator (Osanaga type) and the mushy concentrates were dried from the frozen state.

Extraction of Lipids—The procedures described in the first report (*1*) were generally followed. The lyophilyzed stroma (250 g.) was ground to a fine powder and extracted at room temperature with a mixture of equal parts of pure ether and methanol for 6 days with occasional shaking. Four such extractions were carried out and the residue was filtered on Buchner funnel each time and washed thoroughly with the same solvent. Each extract was evaporated nearly to dryness under reduced pressure, at a temperature not exceeding 50°. After the removal of cholesterol by suction and the filtrate further concentrated *in vacuo*, the residual light yellowish syrup was dissolved in a small amount of anhydrous ether and placed overnight in an ice-box. A white precipitate with waxy appearance (crude sphingomyelin, 5 g.) was removed by decantation. However, in the yellowish ethereal solution, there existed a considerable amount of yellowish-white suspension, which withstood centrifugation. After the addition of a small amount of acetone, the suspension became able to be spun down. The supernatant ethereal solution was reserved for further study. The precipitate was easily soluble in warm methanol and on cooling fine colorless materials separated out. (*Fr. 1*, 2.3 g.), It gave strongly positive Molisch reaction.

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* The substance was obtained by Klenk from bovine spleen (*3*).

The ether-methanol treated residual stroma was air-dried and exhaustively extracted in a Soxhlet apparatus with a mixture of an equal volume of chloroform and methanol. The extract was intensely dark brown. Extraction was continued for 40 hours. The extract was evaporated *in vacuo*, the almost resinous, dark brown residue was dissolved in a small volume of chloroform and precipitated with a large excess of acetone. The amount of precipitate obtained by centrifugation was appreciably small as compared with that obtained in the case of equine stroma (hematoside fraction !). The precipitate was recrystallized from methanol, but only a small amount of colored product was obtained (*Fr. 2*, 0.8 g.).

Analytical Method—Phosphorus was estimated by Allen's method (*5*), iodine number by Yasuda's (*6*), neuraminic or hemataminic acid by Klenk and Langerbeins' (*7*), hexosamine by Blix' (*8*) and reducing value was determined cerimetrically by the method of Brand and Sperry (*9*). Dry combustion methods were used for the estimation of C, H and N and acetyl group by Kuhn and Roth.

Purification of Globoside—*Fr. 1* (P 2.06) and *Fr. 2* (P 1.91, Hexosamine 4.8) were combined (3 g.) and dissolved in warm methanol, to which methanolic solution of cadmium chloride was added until no further precipitate separated. On cooling the warm-filtered solution in an ice-box, colorless precipitate formed. The product was recrystallized three times from methanol (*Globoside*, 0.7 g).

This was colorless, friable powder and fairly hygroscopic. Microscopically it showed considerably homogeneous spherocrystals.

Heated in a capillary tube, the substance turned yellow at 190°, sintered at 200° and melted with decomposition at 224°.

Analysis—

P 0. 47, Sugar (as galactose) 41. 9–43. 6, Hexosamine (as glucosamine HCl) 9. 03–9. 22, Acetyl (after hydrolysis with 50% sulfuric acid for 1 hour) 2. 16. 2. 82.

Dried *in vacuo* over P_2O_5 until constant weight at 110°,

C 64. 08, 64. 30 ; H 10. 25, 10. 39 ; N 2. 09

Optical Rotation—0. 3397 g. of the substance dissolved in pyridine and diluted to to 10 ml., gave, in a 1 dm. tube, $\alpha_D = +0.35°$, hence $[\alpha]_D^{18} = +10.3°$.

Hydrolysis of Globoside:

Fatty Acid Fraction—435 mg. of globoside was dissolved in 30 ml. of 5 per cent methanolic hydrochloric acid in a sealed tube and heated for 3 hours in a boiling water-bath. During hydrolysis, the solution remained colorless. The content of the tube was extracted three times with petroleum ether, which after washing with water, was dried over sodium sulfate, filtered, and evaporated to dryness. The residue weighed 141 mg. (32.4%) and was solid at room temperature. The substance was recrystallized from methanol to colorless plates (30 mg.), melting at 56°, which was converted to the free acid by saponification with methanolic potassium hydroxide. The free acid, recrystallized to colorless plates from petroleum ether, melting at 79–80°, and showed no depression of the melting point on admixture with the authenic sample of lignoceric acid, m.p. 83°.

From the mother liquor of methyl lignocerate, after evaporation of methanol, 104 mg. of femisolid was obtained. The free acid after saponification, was repeatedly recrystallized from aqueous methanol, melting at 36–41°. Iodine number 39. (Nervonic acid, m.p. 40–41°. Iodine number 69).

Base Fraction—After extraction with petroleum ether, methanol was evaporated under reduced pressure, and the residue was made alkaline to phenolphthalein with barium hydroxide and extracted three times with ether. The ethereal extract was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness, leaving 109 mg. (25 per cent) of the crude base as a light brown solid. The solid was acetylated with pyridine and acetic anhydride. The amount of sample was so small that the analytically pure acetate could not be obtained.

Sugar Fraction—After extraction with ether, the aqueous solution was saturated with carbon dioxide to remove the excess of baryt and finally the silver with hydrogen sulfide. The filtrate was concentrated *in vacuo*, leaving 203 mg. (46.7 per cent) of syrup. The syrup was heated with 2 ml. of 5 N HCl for 2 hours on a steam bath. Chlorine ion was removed with silver carbonate and concentrated to about 2 ml. To the solution was added 0.2 g. of freshly distilled α-methyl phenylhydrazine. Light-yellow crystals which separated on cooling overnight in an ice-box, was recrystallized from methanol to colorless plates, m.p. 186–8°. There was no depression of melting point when fused with pure D-galactose-α-methylphenylhydrazone, m.p. 189°

Paper Chromatography—

(a) *Neutral Sugar*—Portions of 1 per cent glucose, galactose and above-mentioned sugar mixture of globoside, each of which contained about 50 μg. of sugar, were placed 2 cm. apart on a horizontal line ruled 7 cm. from the edge of a sheet of Tōyō Roshi No. 2 paper. The sheet was developed for 17 hrs. with a mixture of pyridine, butanol and water (3 : 5 : 1). The paper was then dried at room temperature and sprayed with aniline hydrogen phthalate reagent (*10*) to locate the position of sugars. Similar procedure was tried with the sugar mixture from hematoside. The results are shown in Fig. 2.

(b) *Hexosamine*—About 4 mg. of globoside was hydrolyzed with hydrochloric acid and then evaporated to dryness in a desiccator. 0.2 ml. of water was added and the solution was introduced on a paper strip in the same way as above. As a control, appropriate amount of each glucosamine- and chondrosamine-hydrochloride* was placed.

Solvent used was the same as above. For detection of spots, ninhydrin solution was sprayed. The results are shown in Fig. 3.

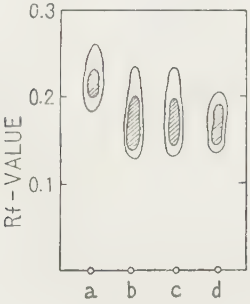

Fig. 2. Paper chromatogram of neutral sugar.

(a) glucose, (b) sugar-mixture of globoside, (c) sugar-mixture of hematoside, (d) galactose.

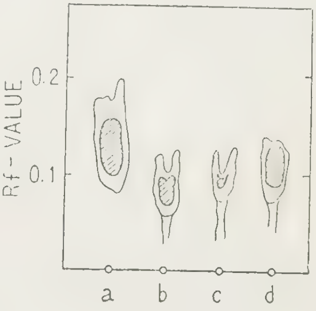

Fig. 3. Paper chromatogram of hexosamine.

(a) glucosamine-HCl, (b) hydrolysate of globoside, (c) hydrolysate of hematoside (in sufficient amount), (d) chondrosamine-HCl.

Carbazole Test :

The procedure employed by earlier workers (*11*, *12*, *13*) was modified as follows.

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* Chondrosamine -HCl was generously supplied by Dr. Z. Yoshizawa of Prof. Masamune's laboratory.

Reagent—Commercial carbozole was purified by washing twice with cold sulfuric acid and recrystallized from toluene and alcohol, sublimated twice and finally recrystallized from absolute alcohol. The carbozole thus purified (30 mg.) was dissolved in 180 ml. of sulfuric acid (8 : 1). The reagent should be colorless and preserved in a refrigerator.

Method—In clear tubes (12×180 mm.) are placed 0.5 ml. of 0.01 per cent solution of glucose and galactose and various proportions of these two sugars. Weighed samples (0.11 to 0.15 mg.) of globoside and hematoside were introduced in other tubes and to these and another blank tubes were added 0.5 ml. of water. 5 ml. of the carbozole reagent were carefully added to each tube under ice-cooling. All tubes were shaken in an ice bath, then placed simultaneously in a vigorously boiling water-bath for exactly 10 minutes. They were then simultaneously removed and cooled in cold water. The coloration was analyzed by the Beckman spectrophotometer at wave-lengths ranging from 400 to 600 mμ (Fig. 4, 5 and 6).

From the spectrophotometric data, ratios of the optical densities of the colored solution at two wave-lengths (540 mμ/440 mμ) were calculated.

Results :	Glucose : galactose	E_{540}/E_{140}
	1 : 0	4. 51
	2 : 1	3. 13
	1 : 1	2. 61
	1 : 3	2. 19
	0 : 1	1. 33
	Globoside	1. 55, 1. 57
	Hematoside	1. 60, 1. 61

Therefore, the content of glucose was extremely smaller than that of galacose in both globoside and hematoside.

The authors wise to express their gratitude to Prof. S. Akiya for encouragement, to Drs. T. Uchida and N. Ogawa for advices.

They also thank Miss R. Ōta, Miss. E. Kondō and Mr. B. Kurihara for C, H and N analyses.

SUMMARY

1. A glycolipid, designated globoside was isolated from lyophilyzed human blood stroma.

2. Globoside contained chondrosamine instead of hemataminic acid.

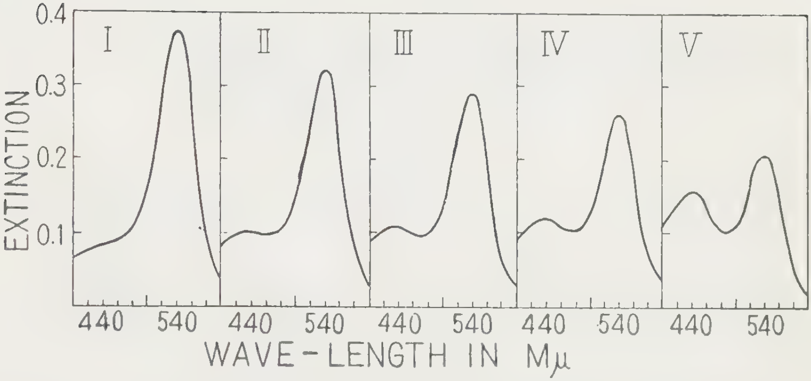

FIG. 4. Absorption curves of carbazole reaction with various mixtures of glucose and galactose. (I) Pure glucose. (II) Glucose 3, galactose 1. (III) Glucose 1, galactose 1. (IV) Glucose 1, galactose 3. (V) Pure galactose.

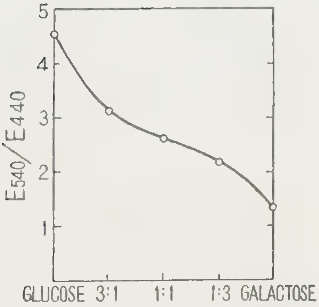

FIG. 5. Ratio of exinctions at 540 mμ and 440 mμ in the absorption curves of Fig. 4.

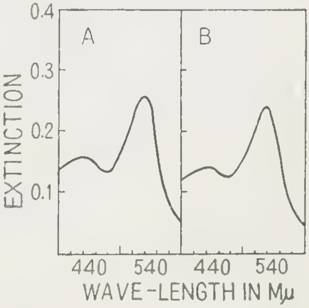

FIG. 6. Absorption curves with globoside (A) and hematoside (B).

3. Glucose was found in both globoside and hematoside but its content was much smaller than that of galactose.

4. The probable structure of globoside was proposed.

ADDENDUM

Recently, an alomst phosphorus-free preparation of globoside was obtained. The analytical data with this sample are as follows:

C 60.42–61.27, H 9.30–9.71, N 2.28, Sugar (as galactose) 52.9–56.2, Hexosamine (as glucosamine HCl) 15.6–15.9, d.p. 240°.

In view of these values, this globoside is assumed most probably to be lignoceryl-(or nervonyl)-sphingosine-acetylchondrosamine-trihexoside.

Calcd. for $C_{63}H_{126}N_2O_{23}$ (1339.71);

C 60.96, H 9.48, N 2.09, Sugar 53.7, Hexosamine 16.1

REFERENCES

(*1*) Yamakawa, T., and Suzuki, S., *J. Biochem.*, **38**, 199 (1951)
(*2*) Klenk, E., and Lauenstein, K., *Z. physiol. Chem.*, **288**, 220 (1951)
(*3*) Klenk, E., and Rennkamp, F., *Z. physiol. Chem.*, **273**, 253 (1942)
(*4*) Yamakawr, T., and Suzuki, S., *J. Biochem.*, **39**, 175 (1952)
(*5*) Allen, R. J. L., *Biochem. J.*, **34**, 868 (1940)
(*6*) Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931-2)
(*7*) Klenk, E., and Langerbeins, H., *Z. physiol. Chem.*, **270**, 185 (1941)
(*8*) Blix, G., *Acta Chem. Skand.* **2**, 467 (1948)
(*9*) Brand, F. C., and Sperry, W. M., *J. Biol. Chem.* **141**, 545 (1941)
(*10*) Partridge, S. M., *Nature*, **164**, 443 (1949)
(*11*) Gurin, S., and Hood, D. B., *J. Biol. Chem.*, **131**, 211 (1939)
(*12*) Seibert, F. B., and Atno, J., *J. Biol. Chem.*, **163**, 511 (1946)
(*13*) Knight, C. A., *J. Exp. Med.*, **85**, 99 (1947)

The Journal of Biochemistry, Vol. 39, No. 4.

THE METABONISM OF DL-ALLOTHREONINE, DL-THREONINE AND DL-METHIONINE IN PHLORHIZIN GLYCOSURIC DOGS AND IN NORMAL FAST RATS

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(Received for publication, July 25, 1952)

The indispensability of threonine and methionine in man and the rat has been demonstrated by Rose and his colaborators. (*1*), (*2*), (*3*), (*4*) However, no satisfactory information on whether or not the amino acid possesses any glycogenic properties has yet been given. In their studies on the rates of absorption and formation of liver glycogen, Hall, Day and Eaton (*5*), and Hess (*6*) succeeded in demonstrating the production of a considerable amount of liver glycogen after administration of threonine and allothreonine. Vars (*7*), in his phlorhizin experiment, found that glucose was formed from methionine in a considerable amount, while Chase and Lewis (*8*), and Hess (*9*) failed to find any increase in liver glycogen after administration of methionine.

The present work is concerned with the glycogenic properties of DL-allothreonine, DL-threonine and DL-methionine in phlorhizin glycosuria in normal fast rats.

EXPERIMENTAL

DL-Allothreonine and DL-threonine were synthesized by Carter's method (*10*) and isolated from each other by utilizing the differnce in solubility between the copper salts of their O-methyl derivatives.

Solubility in water	
DL-O-Methyl-allothreonine copper salt	0.141%
DL-O-Methyl-threonine copper salt	1890%

Purity of each compound was checked by analysis and by the melting points of their *p*-toluensulfonyl derivatives. DL-Methionine was prepared by recrystalization of a certain commercial product and was checked by analysis.

		Nitrogen (per cent)	*p*-Toluensulfonyl derivative
DL-Allothreonine	Calculated	11.76	[196–198°]
	Found	11.72	
DL-Threonine	Calculated	11.76	[178–180°]
	Found	11.63	
DL-Methionine	Calculated	9.40	
	Found	9.26	

In the present experiments, DL-allothreonine and DL-threonine were administered in the form of an aqueous solution of their free acids, while DL-methionine was given as an aqueous solution of its sodium salt, since the solubility of its acid is so low that no concentration sufficiently high for practical use was obtainable.

Experiments on Phlorhizin Glycosuric Dogs—Healthy female dogs were used as experimental subjects. Food was withdrawn from each animal 2 days before the first injection of phlorhizin and the fasting was continued throughout the experiment except the period for administration of the compound under investigation. Water was given *ad libitum.* Each dog received phlorhizin daily by subcutaneous injection in a dose of 0.15 g. per kg. of body weight. The drug was ground and suspended in 7 ml. of olive oil as recommended by Coolen (*11*). The urine was collected every 24 hours and reserved separately under toluene until analyzed. Total nitrogen was determined by Kjeldahl's method, glucose by Bertland's, and acetone bodies by Van Slyke's.

Ten g. of each amino acid was injected subcutaneously. The extra glucose output was calculated by the method described by Rose *et al.* (*12*) The theoretical or expected output was calculated upon the assumption that 2 moles of allothreonine, threonine or methionine was equivalent to 1 mole of glucose. The results of the experiments are summarized in Tables I to III.

In Table I are presented the results of the experiments with DL-allothreonine. In this case the amount of extra glucose found was 1.56 g., that is, 20.6 per cent of its theoretical yield. However, in the urine collected in 24 hours after administration there was found 4.54 g. of unchanged allothreonine as determined by Nicolet and Shinn's method with periodate (*13*), after decomposition of glucose by fermentation. Hence, the theoretical yield had to be corrected by subtracting 4.54 g. from 10 g. Consequently the extra glucose formed was 37.8 per cent of the corrected theoretical yield. Allothreonine in excreted urine showed no optical activity, therefore it was presumably of DL-form. No significant rise in the output of acetone bodies was observed.

TABLE I

Fate of DL-*Allothreonine in Phlorhizin Glycosuria*

Weight	Date	Urine				Extra glucose		
		Glucose	Total N	G : N	Acetone*	Calculated	Found	Conversion
kg.	(1952)	*g.*	*g.*		*g.*	*g.*	*g.*	*per cent*
3.6	Jan. 31	9.25	4.20	2.20	0.037			
	Feb. 1	7.03	3.49	2.01	0.023			
3.3	〃 2	6.53	2.55†	2.56	0.017	7.56 (4.13)	1.56	20.6 (37.8)
	〃 3	2.65	1.62	1.64	0.007			

* Total "acetone bodies" expressed as acetone. † Corrected for the nitrogen content of allothreonine. ( ) Corrected for the allothreonine excreted in urine.

TABLE II

Fate of DL-*Threonine in Phlorhizin Glycosuria*

Weight	Date	Urine				Extra glucose		
		Glucose	Total N	G : N	Acetone*	Calculated	Found	Conversion
kg.	(1952)	*g.*	*g.*		*g.*	*g.*	*g.*	*per cent*
3.8	Feb. 12	11.86	4.16	2.85	5.03			
	,, 13	13.74	5.76	2.39	0.31			
3.5	,, 14	10.13	3.40†	2.98	0.55	7.56 (4.03)	1.22	16.1 (30.3)
	,, 15		Animal died					

* Total "acetone bodies" expressed as acetone. † Corrected for the nitrogen content of the threonine. ( ) Corrected for the threonine excreted in urine.

TABLE III

Fate of DL-*Methinonine in Phlorhizin Glycosuria*

Weight	Date	Urine							Extra glucose		
		Glucose	Total N	G:N	Acetone*	Total S	Inorg SO_4-S	Total SO_4-S	Calculated	Found	Conversion
kg.	(1952)	*g.*	*g.*		*g.*	*g.*	*g.*	*g.*	*g.*	*g.*	*per cent*
4.0	Feb. 26	14.12	5.87	2.41	0.53	0.262	0.258	0.236			
	〃 27	22.16	8.73	2.54	0.45	0.650	0.444	0.385			
3.7	〃 28	16.02	5.53†	2.90	0.53	1.074	0.513	0.484	6.47 (3.85)	2.32	38.4 (60.3)
	〃 29				0.18	0.765	0.189	0.174			

* Total "acetone bodies" expressed as acetone. † Corrected for the ntrogen content of the methionine. ( ) Corrected for the methionine excreted in unine.

In Table II are recorded the results of the experiments with DL-threonine. In this case the extra glucose found amounted to 1.22 g., *viz.*, 16.1 per cent of the theoretical yield. However, in the urine collected in 24 hours after administration 4.67 g. of the amino acid was refound by the periodate method, so that the extra glucose was 30.3 per cent of the corrected theoretical yield. Threonine in the excreted urine was found to be dextro-rotatory after fermentation. This fact means that L-form was utilized in preference to D-form. Calculated from the specific rotation, 1.32 g. of L-form and 3.35 g. of D-form were excreted. No increase in acetone bodies was found.

Table III contains the results of experiments with DL-methionine. Again 2.32 g. of extra glucose, *viz.*, 38.5 per bent of the theoreticaly yield, was found, and in 48 hours' post-administration urine there was 3.63 g. of methionine determine as volatile iodide by Baernstein's method. (*14*) Thus the extra glucose formed was 60.4 per cent of the corrected thPoretical yield. Methionine in the excreted urine had no optical activity in 3 *N* HCl solution. The excretion of S in urine, especially neutral S fraction, was also increased. Acetone bodies were not increased.

Experiments on Fast Normal Rats—Young white rats weighing 120-160 g. afther 18 hors' fast were used. Following this fast, the animals were placed eabh in a separate cage and given an amino acid solution at 2 hours' intervals, first by intraperitoneal injection, then orally through a stomach tube, and finally by intraperitoneal injection again, the amount of the amino acid administered each time being greater than could be adsorbed during the interval. In a control experiment, a 1.0 per cent NaCl solution was given in place of the amino acid solution. At the end of 6 and 9 hours, the animals were killed by a blow on the head, and the livers were removed and weighed as quickly as possible. After that they were submitted to glycogen determination by Good, Kramer and Somogyi's method (*15*). The precipitated glycogen was hydrolyzed with hydrochloric acid and the glucose was determined by the micro-Bertrand's method. As much urine as possible was collected, and the amino acid was checked by paper chromatography and by measuring optical activity.

A small but significant increase in liver glycogen was confirmed after each amino acid was given. The results are listed in Table IV.

Since the values of liver glycogen after administration of DL-allo-threonine and DL-threonine were slightly less increased in the present experiment that reported by Hall *et ol* (*5*) and Hess (*6*), DL-alanine

TABLE IV
Glycogen Content in Rats Killed 6 or 9 Hours after the Administration of Amino Acid

Amino acid	No. of rat	Absorption duration in hours	Glycogen content	The amount of the amino acid given per 100 *g.* body weight per hour
		hours	*per cent*	*mg.*
Control (1.0% NaCl solution)	5	6–9	0.07	
DL-Allothreonine	3	6	0.27	100
DL-Threonine	3	6	0.29	100
DL-Methinonine	4	6	0.12	50
//	3	9	0.26	50
DL-Alanine	2	6	0.29	100

was tested in the same manner as in the control experiment. In this case the increase of liver glycogen was again slight. This small extent of glycogen formation might be due to the low room temperature (5°) at which the experiment was made. Hess and Chase *et al.* reported that no significant increase of liver glycogen was observed 3 hour after administration of DL-methionine. In the present experiment, a small but significant increose of liver glycogen was found 6 hours after the administration, and the increase became more evident at the end of 9 hours. The conclusion may be drawn from the result that liver glycogen is formed from DL-methionine more slowly than from other amino acids. Each amino acid given was detected in the excreted urine afterward, and more D-form than L-form was found after administration of DL-threonine, while this was not the case after application of their DL-allothreonine or DL-methionine. These results are quite in accord with the experiments with phlorhizinized dogs.

SUMMARY

1. In phlorizinized dogs, DL-allothreonine, DL-threonine and DL-methionine were all capable of yielding glucose.

2. The amino acids administered to fast normal rats were transformed into liver glycogen though the amount of glycogen was not so large but significant 6 or 9 hours after its administration.

3. In the urines of dogs and rats a certain amount of the administered amino acid was excreted. In the case of DL-allothreonine and

DL-methionine, the racemic form, while in that of DL-threonine more D-form than L-form was found.

The auther wishes to thank Prof. R. Hirohata for his advice and encouragement during this study.

REFERENCES

(*1*) Meyer, C. E., and Rose, W. C., *J. Biol. Chem.*, **115**, 721 (1936)
(*2*) Womack, M., Kemmerer, K. S., and Rose, W. C., *J. Biol. Chem.* **121**, 403 (1937)
(*3*) Rose, W. C., Haines, W. J., Warner, D. T., and Johnson, J. E., *J. Biol. Chem.*, **188**, 49 (1951)
(*4*) Rose, W. C., Johnson, J. E., and Haines, W. J., *J. Biol. Chem.*, **182**, 541 (1950)
(*5*) Hall, W. K., Doty, J. R., and Eaton, A. G., *Am. J. Physiol.*, **131**, 252 (1940)
(*6*) Hess, W. C., *J. Au. Chem. Soc.*, **72**, 1407 (1950)
(*7*) Vars, H. M., *Proc. Soc. Exp. Biol. Med.*, **31**, 129 (1933-34)
(*8*) Chase, B. W., and Lewis, H. B., *J. Biol. Chem.*, **101**, 735 (1933)
(*9*) Hess, W. C., *J. Biol. Chem.*, **181**, 23 (1949)
(*10*) Carter, W., *J. Biol. Chem.*, **119**, 103 (1937)
(*11*) Coolen, F., *Arch. Pharmacol.*, **1**, 267 (1895)
(*12*) Rose, W. C., *J. Biol. Chem.* **145**, 679 (1942)
(*13*) Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **138**, 91 (1941)
(*14*) Baernstein, H. D., *J. Biol. Chem.*, **115**, 25 (1936)
(*15*) Kramer, H., Good, C. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933)

ABSTRACTS

from

The Journal of Japanese Biochemical Society (Seikagaku).
Volume 23 (1951) p. 239-264 and Volume 24 (1952) p. 25-73.

27. Aneurinase. XVI. The properties of bacterial aneurinase and its activation by basic organic compounds. Toshio Tashiro (Kyoto Prefect. Med. Coll.) p. 239–244. The activity of bacterial aneurinase has the optimum temp. at 30°; it is completely inactivated by heating at 100° for 20 min.; the enzyme is specific to thiamine, but slightly active for 2-ethyl-thamine. NaF inhibits aneurinase, but Cd^{++} and Cu^{++} rather accelerate it. The acceleration of the enzyme is also induced by aniline, quinoline, pyridine, pyrimidine, and their derivatives substituted at the other groups than aromatic -N or -NH_2, and some thiol compounds. Aliphatic NH_2-compounds give no effect on the enzyme activity.

28. On the composition of serum protein, especially the fluctuation of γ-globulin fraction in some infantile diseases. Mitsuhiko Matsuda (Segawa Ped. Hosp., Tokyo). p. 244–249.

29. Aneurinase. XVII. The transferring reaction of pyrimidine group of thiamine by aneurinase. Yoshizugu Nose (Kyoto Prefect. Med. Coll.). p. 249–253. By the action of shell-fish aneurinase (the extracts of clam) on a mixture of thiamine and pyridine at pH 5.5, 50° for 1 hr., there were formed HPT (heteropyrithiamine: 2-methyl-4-amino-5-pyrimidylmethylpyridinum) together with the liberated thiazole part of thiamine 4-methyl-5-β-hydroxy ethyl-thiazole). The enzyme also catalyzed the formation of quino-thiamine from a mixt. of thiamine and quinoline. By ferricyanide oxidation in alkaline solution, HPT and quinothiamine were further transformed to fluorescent substances, pyrichrome and quinochrome, respectively. The enzyme did not catalyze the synthetic reaction between pyrimidyl-methyl-alcohol and either pyridine or quinoline. The marked acceleration of aneurinase activity by pyridine or quinoline is interpreted to by attributed to its transferring reaction.

30. Ibid. XVIII. On the synthesis of thiamine by aneurinase. Yoshizugu Nose. (ibid.) p. 253–259. The synthesis of thiamine or its derivatives has been studied by the incubation of a mixture of aneurinase preparation, pyrimidin derivative (0.001 *M*) and 4-methyl-5-β-hydroxyethylthiazol. (a) Shell-fish aneurinase has the optim. conditions at pH 4.0 and at 70° for the synthetic reaction. The yield of thiamine derivatives at 50°, pH 4.0 with various pyrimidine derivatives is as follows: N-(2-methyl-4-amino-pyrimidyl-5-methyl)-pyridinum bromide · HBr (I)$>$N-(2-methyl-4-amino pyrimidyl-5-methyl)-quinolinum bromide · HBr (II)$>$N-(2-methyl-4-amino-pyrimidyl-5-methyl)-*m*-nitroaniline · HBr. (b) Bacterial aneurinase shows the optim. temp. at 30–35°, and the optim. pH at 4.0. The yield of the synthetic reaction: II$>$I. (c) Fish aneurinase has the optimum pH at 4.0 and the optimum temp. at 38°. I and II are available effectively for the synthesis. Thiamine synthesis by aneurinase preparations may indicate the occurrence of the reverse reaction of the transferring action of this enzyme (cf., the preceeding paper, *Aneurinase*, *XVII*.)

31. The decomposition of vitamin A by various factors. III. On the influence of inorganic salts upon the decomposition of vitamin A under the irraditation of ultrasonic waves. Kaizo Ariga (Nippon Univ. Med. Sch., Tokyo) p. 260–264; cf., this *Abstracts*, **21**, 76 (1948); **22**, 78 (1949). Ultrasonic wave irradiation (560 k. cycle) resulted in neither vitamin A decomposition nor peroxide formation. When ground pieces of glass or rock-crystal were present, about 90 and 70% of vitamin A were decomposed, respectively; and at the same time, the temperature rose to 65 and 60° in 30 min., respectively, accompanying the production of peroxide. The similar results were attained by irradiating vitamin A in a test tube which is placed in glass or rock-crystal pieces. Such an effect was obtained with inorganic salts in solid state, such as NaCl, SiO_2, $CaCl_2$, KCl, and Na_2SO_4. Under anaerobic condition vitamin A was not decomposed. The effect of ultrasonic wave irradiation upon vitamin A decomposition may mainly be due to the formation of heat by the action of the waves on glass, rock-crystal, or inorganic salts.

Volume 24 (1952)

1. Aneurinase. XIX. The decomposition of thiamine derivatives by aneurinase. Yoshihzugu Nose (Kyoto Prefect. Med. Coll.) p. 25–29. A method has been devised for the determination of HPT (heteropyrithiamnie) in the presence of thiamine by decomposing thiamine with 30% NaOH. The procedure is as follows: The eluate of permutite-adsorbed sample is heated with 30% NaOH at 45° for 30 min. After colling, 0.1–0.4 ml. of 1% ferricyanide are added, and the mixture is left standing for 20 min. The formed pyrichrome is determined fluorometrically. Shellfish aneurinase decomposes thiamine and HPT at the same rate, but bacterial aneurinase is 3 times active for thiamine as much as for HPT.

2. Studies on the mechanism of the oxidation of ascorbic acid. I. On the in fluence of pyridine upon the oxidation of ascorbic acid catalyzed by copper ions. Taro Tomimura (Nippon Med. Coll., Tokyo) p. 29–33. The rate of the oxidation of $2\times10^{-5}\,M$ ascorbic acid at pH 4.3 (acetate buffer) catalyzed by Cu^{++} increases with the increasing Cu^{++} concentration. The addition of pyridine lowers the oxidation rate and prolongs the induction period. The oxidation rate of ascorbic acid in the presence of a constant amount of pyridine ($0.25\times10^{-3}\,M$) increases in proportion to Cu^{++} concentration.

3. Ibid. II. On the effect of H_2O_2 on the oxidation of ascorbic acid catalyzed by Cu-pyridine system. Taro Tomimura., p. 33–36. With a reaction mixture consisting of $2\times10^{-5}\,M$ ascorbic acid, $0.2\times10^{-6}\,M$ Cu and $0.25\times10^{-3}\,M$ pyridine at pH 4.3 (acetate buffer), the rate of oxidation of ascorbic acid increases rapidly on adding H_2O_2 to the reaction mixture. When H_2O_2 is contained in the starting reaction mixture, the oxidation rate increases in proportion to H_2O_2 concentration, but O_2-uptake decreases which may be due to the peroxidatic oxidation of ascorbic acid by the Cu^{++}-pyridine complex compound. An excess amount of pyridine inhibits both the decomposition of ascorbic acid and O_2-uptake.

4. **Enzymatic hydrolysis of β-cellobioside. I. On the specificity of β-cellobioside hydrolyzing enzymes in apricot and malt emulsin.** Kazutoshi Nishizawa and Kazumasa Wakabayashi, p. 36–43. (Shinshu Univ., Suwa, Nagano). The hydrolysis of substrates by enzyme preparations from apricot emulsin was determined by measuring reducing power and the liberated *p*-nitrophenol. These preparations acted strongly on *p*-nitrophenol-β-glucoside, slightly on *p*-nitrophenol-β-cellobioside, and far less on β-cellobiose. The comparison of the reducing power increments (calculated as glucose) with the amounts of liberated *p*-nitrophenol indicates that apricot emulsin contains β-glucosidase, but neither specific β-cellobiosidase nor β-cellobiase. This β-glusidase acts also as β-cellobiosidase, liberating 2 moles glucose from 1 mole substrate, which is hydrolyzed to aglucon and pheron, where the holloside linkageis more easily split off than the cellobioside bond. By use of enzyme preparations of malt emulsin the hydrolysis of substrates is greater in the following order : β-cellobiose$\gg$*p*-nitrophenol-β-glucoside $>$*p*-nitrophenol-β-cellobioside. This malt emulsin contains evidently the specific β-cellobiase and a slight amount of β-glucosidase, but not specific β-cellobiosidase. The evaluation of the changes in the liberated *p*-nitrophenol and reducing power manifests itself that the observed hydrolysis of β-cellobioside is brought about by the action of the contaminated β-glucosidase.

5. **Aneurinase. XX. The values of Michaelis constant of aneurinase reaction.** Akiji Fujita and Yoshizugu Nose (Kyoto Prefect. Med. Coll.) p. 44–49. By use of a glass stoppered tube equipped with 2 side arms, the velocity of aneurinase reaction with thiamine-chloride was measured at pH 5.5 by in adding 10% metaphosphoric acid to the reaction mixtured from a side arm. The following values of Michaelis constant were obtained : (a) Shell-fish aneurinase of clam extracts (1 : 10); 0.6×10^{-6} mole/lit. at 40°; (b) Fish aneurinase of crucian carp and carp at 40°, 2.5×10^{-6} and 0.32×10^{-6} mole/lit. with liver extract (1 : 20), respectively, and 6.7×10^{-6} and 1.0×10^{-6} mole/lit. with kidney extract (1 : 20), respectively; (c) Bacterial aneurinase of the diluted medium of *Bac. thiaminolyticus*, 0.3×10^{-6} mole/lit. at 38°.

6. **Ibid. XXI. Activation energy of aneurinase reaction.** Akiji Fujita and Yoshizugu Nose, (ibid.) p. 50–51. From the rate of thiamine decomposition by aneurinase at different temperatures estimated with a tube with 2 side arms, the activation energy was calculated as follows : (a) Shell-fish aneurinase, 5.5 (20–30°), 4.3 (30–40°), and 2.7 (40–50°) k. cla./mole; (b) Fish aneurinase of crucian carp liver and kidney, 8.4 (20–30°), 8.3 (30–40°), and 3.1 (40–50°) k. cal./mole : (c) Bacterial aneurinase, 11.6 (20–30°) and 9.0 (30–40°).

7. **Studies on the isomerism of coproporphyrin. I. A method for the determination of isomeric coproporphyrins by use of partition paper chromatography.** Shimpachi Nishikawa (Inst. Pub. Health, Tokyo Univ.) p. 52–54. A method has been devised

for the analysis of isomeric coproporphyrins contained in urine. The procedure is as follows: To 5 ml. of urine add 1 ml. of glacial acetic acid, and 5 drops of 30% H_2O_2, successively. Mix well with 10 ml. of ethylether, wash the ethylether layer with 0.5 ml. of 0.5% HCl. Place 0.1 ml. of the HCl-extract on a strip of filter paper (Toyo Roshi No. 2, 2.5 cm.×40 cm.), dry rapidld at 40°, cool to room temp., and develop with methanol. Just on ascending the solvent boundary beyond 30 cm., detect the pink or red fluorescent spots of coproporphyrin under ultraviolet ray, and measure the rate of flow. Normal urine contains only copropophyrin I (cpr. I) and III (cpr. III), R_f-values of which are found to be 0.00 ± 0.01, and 0.17 ± 0.08, respectively. These values are shifted by the contaminated substances in urine. The standard R_f-values of pure cpr. I and III are 0.00 ± 0.01 and 0.14 ± 0.04, respectively. By this method 0.5 γ cpr. I/dl., and 1.8 γ cpr. III/dl. are detectable.

8. Studies on the function and metabolism of hemoglobin. II. The decomposition of hemoglobin by hydrogen peroxide. Gorō Kikuchi (Nippon Med. Coll., Tokyo) p. 55–62. With a mixture of catalase-free O_2Hb prepared from blood of *Anenzymia catalatica* and H_2O_2, the conversion of O_2Hb into choleglobin reaches an equilibrium, with a yield of 30%. The formation of byproduct, possibly propentdyopent, increases more greatly than that of choleglobin with the increase in H_2O_2 used. O_2Hb having denatured globin used by NaOH treatment is scarcely converted to choleglobin by the action of H_2O_2. MetHb can be transformed to choleglobin by H_2O_2 only after reducing MetHb to Hb with $Na_2S_2O_4$. Ascorbic acid gives no effect on this choleglobin forming-reaction by H_2O_2.

9. Ibid. III. On the reaction of hemoglobin-KCN-ascorbic acid system. Goro Kikuchi (ibid.). p. 62–67. The formation of choleglobin has been studied with a reaction mixture containing 0.20% O_2Hb, 0.1 *M* KCN and 0.01 *M* ascorbic acid. With the increase in ascorbic acid the initial reaction rate is accelerated, but the yield of choleglobin is limited to 78% in all cases. In 0.1–0.01 *M* KCN, choleglobin is produced twice as much as that in the absence of KCN; but higher concentration of KCN (0.05–0.1 *M*) rather lowers the initial rate of choleglobin formation.

10. Ibid. IV. On the pseudo-hemoglobin forming reaction. Gorō Kikuchi and Mitsuru Horiguchi (ibid.). p. 67–70. By the simultaneous action of KCN and H_2O_2 on catalase-free O_2Hb, there are formed CN-metHb together with pseudo-Hb. Both the reaction rate and the yield of pseudo-Hb rise with the increasing H_2O_2 at a constant KCN concentration, *viz.* with the increasing KCN in the presence of ascorbic acid at a constant concentration. But the excess H_2O_2 rather promotes the side reaction to form met-Hb, which further reacts with CN^-.

11. Ibid. V. On the interrelation between choleglobin formation and ascorbic acid oxidation. Goro Kikuchi and Takashi Ogawa (ibid.). p. 70–73. A mixture of 5 ml. of 0.6% O_2Hb, 5 ml. of M/15 phosphate buffer (pH 7.38) and 2.2–52.8 mg. of ascorbic acid was incubated at 37.5° for 5–7 hrs. By use of catalase-free O_2Hb, both the rate and the yield of choleglobin formation increase in proportion to the amount of ascorbic acid used. But in case of O_2Hb from normal blood containing catalase, the amount of formed choleglobin increase at approx. constant rate in the presence of more than 4.4 mg. of ascrbic acid; the initial lag phase is shortened in parallel with ascorbic acid concentration; and the decomposition of ascorbic acid, directly determined by iodometric titration, proceeds at higher rate than the choleglobin formation. For the formation of 1 mole choleglobin 20, moles ascorbic acid are calculated to be consumed. The reaction: $O_2Hb \rightarrow$ choleglobin requires H_2O_2, formed by the oxidation of ascorbic acid, where H_2O_2 can possibly be catalyzed by the intermediate compounds in this reaction of choleglobin formation from O_2Hb.